IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of: Docket No.: 06510003PB

Marshall Schwartz

Serial No.: 09/931,112 Group Art Unit: 1631

Filed: August 17, 2001 Examiner: M. BORIN

For: **TREATMENT OF INTESTINAL EPITHEIAL CELL MALFUNCTION, INFLAMMATION OR DAMAGE WITH HEPATOCYTE GROWTH FACTOR**

DECLARATION UNDER 37 C.F.R. § 1.131

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

I, Marshall Z. Schwartz, M.D., declare the following:

1. I am the inventor of the above-identified patent application, Serial No. 09/931,112.

2. The above-identified application is a divisional application of U.S. Application Serial No. 09/395,129, filed on September 14, 1999, now U.S. Patent No. 6,319,899, which in turn is a continuation-in-part application of U.S. Application Serial No. 08/932,391, now U.S. Patent No. 5,972,887, which claims priority to provisional application Serial No. 60/026,352, filed on September 19, 1996. I am the inventor of the subject matter disclosed in these applications as well.

3. I have read and I am familiar with the Office Action mailed July 26, 2004 pertaining to this application.

4. I understand that in the Office Action mailed July 26, 2004 the Examiner has rejected claims 7, 9-12 and 21-33 allegedly being obvious over Zushi et al., 270(5 Pt 1) AM. J. PHYSIO., G757-62 (1996) ("Zushi"), in view of Ishii, Japanese Patent Application No. 8-231418 ("Ishii"), and in further view of Fukamachi et al., 205(2) BIOCHEM. BIOPHYS. RES. COMMUN., 1445-51 (1994) ("Fukamachi"), and Halttunen et al., 111(5) GASTROENTEROLOGY 1252-62 (1996) ("Halttunen"). Copies of the references are attached as Exhibits A-D, respectively. (Exhibit B is a translation of the Japanese Application.)

5. I have read and I am familiar with Zushi, Ishii, Fukamachi and Halttunen. It is my understanding that Zushi has a publication date of May 1996, *see* Exhibit A, Ishii has a publication date of September 10, 1996, *see* Exhibit B, and Halttunen has a publication date of November 1996, *see* Exhibit D.

6. Attached as Exhibit E is a copy of an abstract I authored with the assistance of my research assistants, who were working under my direct control and supervision in my research laboratory. The abstract was submitted to the American Academy of Pediatrics Section on Surgery before May 1996. The abstract, which was not publicly disclosed more than one year prior to the September 19, 1996 filing date of provisional application Serial No. 60/026,352, evidences that I reduced to practice in the United States before May 1996 the inventions disclosed and claimed in the present application Serial No. 09/931,112, including the presently rejected claims 7, 9-12 and 21-33.

7. The experimental data and information summarized in the abstract is contemporaneous evidence of the reduction to practice of the claimed inventions, which occurred in the United States before May 1996, the earliest publication dates of the Zushi, Ishii and Halttunen references.

8. In particular, the abstract summarizes a study done in the United States designed to examine the effect of systemically administered HGF on intestinal mass and function in adult male Sprague-Dawley rats. *See* Exhibit E. Specifically, jugular venous catheters

connected to subcutaneously placed osmotic minipumps were positioned within the adult rats. The rats were divided into 4 groups based upon the contents in the osmotic minipumps, i.e., Group 1 (control), Group 2 (HGF 75 μg/kg/d), Group 3 (HGF 150 μg/kg/d). and Group 4 (HGF 300 μg/kg/d). Following a 14 day infusion, mucosal DNA content and protein content were analyzed in a segment of the mid small intestine for each group. The results of the study demonstrated for the first time that HGF can stimulate cell proliferation of intestinal epithelial cells *in vivo*. Accordingly, the results of the study evidenced that HGF stimulated intestinal epithelial cell proliferation *in vivo* before the publication date of the Zushi, Ishii and Halttunen references.

9. All statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that the statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and such willful false statements may jeopardize the validity of the application or any patents issuing thereon.

Marshall Z. Schwartz

Inventor: Marshall Z. Schwartz, M.D.

Date 1-23-05

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☐ 1: Am J Physiol. 1996 May;270(5 Pt 1):G757-62. Related Articles, Li

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Role of prostaglandins in intestinal epithelial restitution stimulated by growth factors.

Zushi S, Shinomura Y, Kiyohara T, Minami T, Sugimachi M, Higashimoto Y, Kanayama S, Matsuzawa Y.

Second Department of Internal Medicine, Osaka University Medical School, Suita, Japan.

Mucosal integrity is reestablished after superficial injuries by a rapid resealin process, termed epithelial restitution, that is regulated by several growth fact and cytokines. Growth factors are also known to stimulate the synthesis of endogenous prostaglandins that mediate important functions in intestinal epithelial cells. Therefore, we examined the effect of endogenous eicosanoid production modulators, piroxicam, dexamethasone, and nordihydroguaiaretic acid (NDGA) on intestinal epithelial restitution using two cultured cell woun resealing models, IEC-6 and Caco-2 cells. Epidermal growth factor, transforming growth factor-beta, hepatocyte growth factor, and fetal calf ser (FCS) accelerated intestinal epithelial restitution, and piroxicam significantly suppressed these stimulatory effects. Dexamethasone mimicked the action of piroxicam. No additive effect of piroxicam and dexamethasone was observe NDGA did not affect epithelial restitution. Piroxicam abolished the increase 6-ketoprostaglandin F1 alpha (PGF1 alpha) release induced by FCS. Furthermore, addition of a stable PGI2 analogue, OP-41483 [5(E)-6,9-deoxa-6,9-methylene-15-cyclopentyl-16,17,18,19,20-pen tanor-PGI2], reversed the slowing of epithelial restitution induced by piroxicam. These results suggest that endogenous prostaglandins play an important role in regulating intestina epithelial restitution.

PMID: 8967486 [PubMed - indexed for MEDLINE]

Role of prostaglandins in intestinal epithelial restitution stimulated by growth factors

SHINICHIRO ZUSHI, YASUHISA SHINOMURA, TATSUYA KIYOHARA, TAKESHI MINAMI, MASAMITSU SUGIMACHI, YOSHIFUMI HIGASHIMOTO, SHUJI KANAYAMA, AND YUJI MATSUZAWA

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Zushi, Shinichiro, Yasuhisa Shinomura, Tatsuya Kiyohara, Takeshi Minami, Masamitsu Sugimachi, Yoshifumi Higashimoto, Shuji Kanayama, and Yuji Matsuzawa. Role of prostaglandins in intestinal epithelial restitution stimulated by growth factors. *Am. J. Physiol.* 270 (*Gastrointest. Liver Physiol.* 33): G757–G762, 1996.—Mucosal integrity is reestablished after superficial injuries by a rapid resealing process, termed epithelial restitution, that is regulated by several growth factors and cytokines. Growth factors are also known to stimulate the synthesis of endogenous prostaglandins that mediate important functions in intestinal epithelial cells. Therefore, we examined the effect of endogenous eicosanoid production modulators, piroxicam, dexamethasone, and nordihydroguaiaretic acid (NDGA) on intestinal epithelial restitution using two cultured cell wound-resealing models, IEC-6 and Caco-2 cells. Epidermal growth factor, transforming growth factor-β, hepatocyte growth factor, and fetal calf serum (FCS) accelerated intestinal epithelial restitution, and piroxicam significantly suppressed these stimulatory effects. Dexamethasone mimicked the action of piroxicam. No additive effect of piroxicam and dexamethasone was observed. NDGA did not affect epithelial restitution. Piroxicam abolished the increase in 6-ketoprostaglandin $F_{1\alpha}$ ($PGF_{1\alpha}$) release induced by FCS. Furthermore, addition of a stable PGI_2 analogue, OP-41483 [5(*E*)-6,9-deoxa-6,9-methylene-15-cyclopentyl-16,17,18,19,20-pentanor-PGI_2], reversed the slowing of epithelial restitution induced by piroxicam. These results suggest that endogenous prostaglandins play an important role in regulating intestinal epithelial restitution.

epithelial injury; wound resealing; prostacyclin

INTESTINAL EPITHELIAL CELLS form a barrier to many luminal irritants, including secondary bile acids, short-chain fatty acids, and bacterial metabolites. This epithelial barrier has been recently known to recover within hours when disrupted by a superficial epithelial injury by a rapid wound healing process called restitution, and progression of the destructive inflammatory process is thus prevented (14, 15).

Epithelial restitution is mainly accomplished by epithelial cell migration that reseals the wound area without any cell proliferation, especially during the initial step of healing. In vitro models using cultured epithelial cells have contributed to the study of epithelial restitution. Fetal calf serum (FCS) and several growth factors including transforming growth factor-α (TGF-α), epidermal growth factor (EGF) (1, 6), TGF-β (4), and hepatocyte growth factor (HGF) (5, 18) have been reported to accelerate intestinal epithelial restitution. Endogenous eicosanoids have been recently reported to mediate intracellular mitogenic signals in response to TGF-α and 12-*O*-tetradecanoylphorbol [illegible] acetate in the intestinal cell line, RIE-1 (8). However, how endogenous eicosanoids affect intestinal epithelial restitution is not known.

In the present study, we examined the role of [illegible]noids in the regulation of intestinal epithelial restitution using two well-characterized intestinal epithelial cell lines, IEC-6 and Caco-2. IEC-6 is a cell line derived from crypt cells of normal rat small intestine and [illegible] been frequently used in studies of proliferation and restitution (7, 16, 17). Caco-2 is a cell line derived from a human colon cancer. Caco-2 is known to differentiate into cells that have the function of epithelial cells [illegible] small intestine (20). This cell line has been extensively studied as a model of differentiated normal intestinal epithelium in examinations of the mechanisms of [illegible]sorption, electrolyte transport, barrier formation [illegible] restitution (1, 10, 11).

MATERIALS AND METHODS

Reagents. Piroxicam and EGF were purchased from [illegible] (St. Louis, MO). Dexamethasone, mitomycin C (MMC), HGF and TGF-β were obtained from Wako Pure Chemicals (Osaka, Japan). Nordihydroguaiaretic acid (NDGA) was purchased from Biomol (Plymouth, PA). A potent stable prostacyclin analogue, OP-41483 [5(*E*)-6,9-deoxa-6,9-methylene-15-[illegible]pentyl-16,17,18,19,20-pentanor-prostaglandin I_2], was a kind gift from Ono Pharmachemical (Osaka, Japan).

Cell culture. The human colon cancer cell line Caco-2 [illegible] rat intestinal cell line IEC-6 were obtained from the American Type Culture Collection (Rockville, MD). Caco-2 [illegible] were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated FCS (Whittaker Bioproducts, Walkersville, MD), 1% nonessential amino acids, 100 U/ml penicillin, and 100 μg/ml of streptomycin. IEC-6 cells were in *passages 23–27* and were routinely cultured in DMEM supplemented with 10% FCS, 100 U/ml penicillin, and 100 μg/ml of streptomycin. Cells were cultured in a water-saturated atmosphere with 5% CO_2 at 37°C.

Wound assay. Cells were harvested by trypsinization [illegible] seeded at 6×10^4 cells/ml in 12-well tissue culture plates (Costar, Cambridge, MA). Cells were further incubated [illegible] days to reach confluency. Caco-2 cells at 7 days after confluency and IEC-6 cells at 3 days after confluency were used [illegible] wound assays. The assays were performed as previously described by Sato and Rifkin (23) with slight modifications. Briefly, IEC-6 cells were treated with 5 μg/ml MMC [illegible] Caco-2 cells were treated with 20 μg/ml MMC for 2 h [illegible] contribution by cell proliferation. The optimal dosage of MMC was determined for the concentration that inhibited [illegible] of each cell completely without affecting cell viability [illegible] estimating [^{3}H]thymidine uptake. As reported by others [illegible] 6), mitosis blockade did not affect restitution of both [illegible]. Next, the medium of the confluent monolayers was changed to fresh serum-deprived medium (DMEM with 0.1% FCS) [illegible]

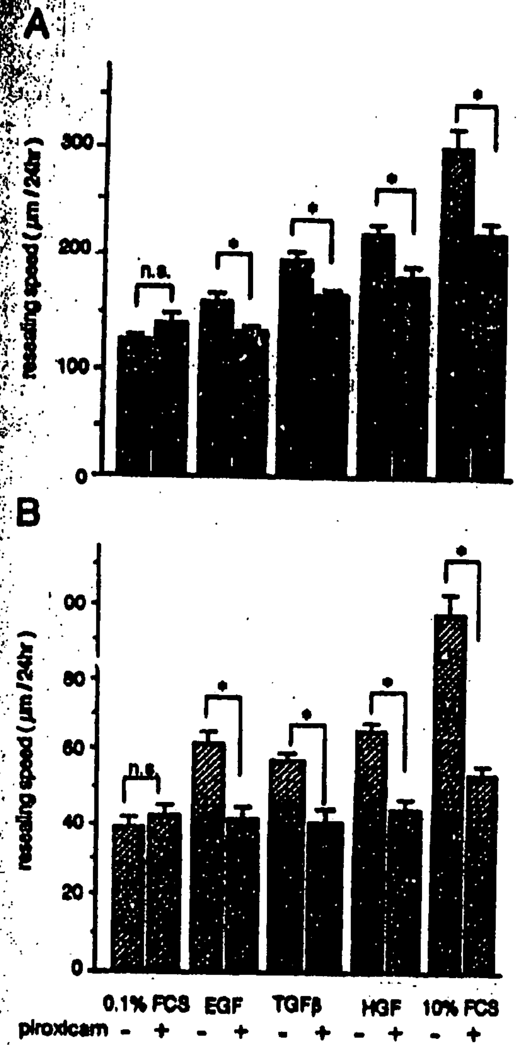

Fig. 1. Epidermal growth factor (EGF), transforming growth factor-β (TGF-β), hepatocyte growth factor (HGF), and fetal calf serum (FCS) accelerated epithelial restitution of IEC-6 (*A*) and Caco-2 (*B*) cells, and piroxicam attenuated these accelerations. Cells were exposed for 2 h to 5 μg/ml mitomycin C (MMC) for IEC-6 and 20 μg/ml MMC for Caco-2 to inhibit mitosis. Medium was changed to serum-deprived medium 6 h before wounding with a razor blade. Medium was then changed to fresh medium containing each reagent with or without 5 μM piroxicam, and cells were further incubated for 24 h. Resealing speed was calculated by dividing the area cells covered over wound edge by wound width. Data are expressed as means ± SE (n = 6). *P < 0.01 compared with resealing speed without piroxicam; n.s., not significant.

before wounding. Inhibitors were added 4 h before wounding. Wounds 8–10 mm in length were made with a razor blade in each well. The cells were then washed with fresh serum-deprived medium, changed to fresh medium supplemented with inhibitors again, and further incubated for 24 h in the presence or absence of the stimulants of interest. The area of the cell sheet that migrated over the w[illegible] measured using a digital image process[illegible] (FLOVEL, Tokyo, Japan) connected to an [illegible] microscope (Olympus, Tokyo, Japan). Resealing [illegible] calculated by dividing the cell sheet area that m[illegible] the wound edge over 24 h by the width of th[illegible] Resealing speed was assessed in a blind fashio[illegible] observer bias.

Assay for 6-ketoprostaglandin $F_{1\alpha}$. Cells were ha[illegible] trypsinization and seeded at 6 × 10^4 cells/ml in 12-[illegible] culture plates. Cells were further incubated fo[illegible] reach confluency. Caco-2 cells at 7 days postconfl[illegible] IEC-6 cells at 3 days postconfluency were used. The [illegible] of confluent monolayers was changed to fresh serum-[illegible] medium and incubated for 2 h. Piroxicam, dexameth[illegible] both were added to the medium and incubated [illegible] Medium was changed to fresh medium containing th[illegible] reagents and further incubated 24 h. The medium w[illegible] collected and the 6-ketoprostaglandin $F_{1\alpha}$ (6-ket[illegible]) concentration was assessed by enzyme-linked im[illegible] bent assay (ELISA; Cayman Chemical, Ann Arbor, MI[illegible]

MTT assay. Cells were trypsinized, seeded at [illegible] cells/ml into a 96-well tissue culture plate and incub[illegible] fresh medium under an atmosphere of 5% CO_2–95% ai[illegible] for 24 h. Medium was then changed to fresh medium con[illegible] ing each reagent at the indicated concentration and furth[illegible] incubated. After 24 h, MTT [3-(4,5-dimethylthiazol[illegible]

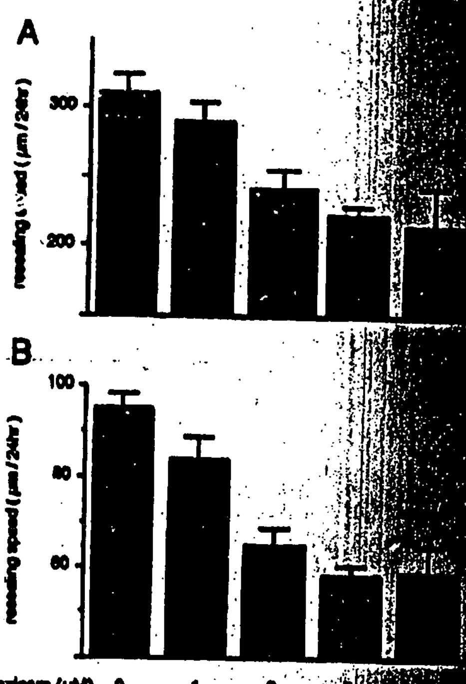

Fig. 2. Effect of piroxicam on restitution of IEC-6 (*A*) and Ca[illegible] cells. Cells were exposed to MMC for 2 h to inhibit mito[illegible] was changed to serum-deprived medium 6 h before [illegible] Piroxicam at each concentration was added 4 h before [illegible] After wounding, medium was changed to fresh medium [illegible] mented with 10% FCS containing same concentration of pi[illegible] before. Cells were further incubated for 24 h, and resealin[illegible] determined. Data are expressed as means ± SE (n = 6).

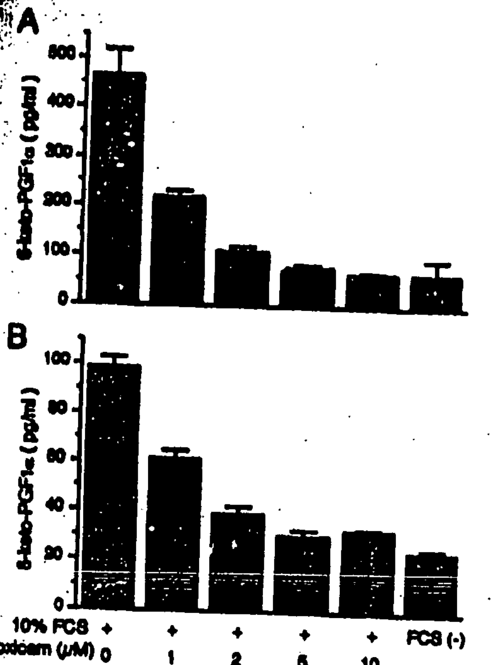

Fig. 3. Production of 6-ketoprostaglandin $F_{1\alpha}$ ($PGF_{1\alpha}$) by IEC-6 (*A*) and Caco-2 (*B*) cells. Medium of confluent cell monolayers was changed to fresh serum-deprived medium, and cells were incubated for 3 h. Medium was changed to fresh medium containing piroxicam at each concentration with 10% FCS or serum-deprived medium, and cells were incubated for 4 h. After that, medium was changed to fresh medium containing piroxicam at each concentration with 10% FCS or serum-deprived medium again, and cells were further incubated for 24 h. Medium was collected and 6-keto-$PGF_{1\alpha}$ concentration was determined by enzyme-linked immunosorbent assay. Results are expressed as means ± SE (n = 3).

diphenyltetrazolium bromide] (Chemicon, Temecula, CA) was added to each well at 500 μg/ml and incubated for 4 h. The formazan produced was solubilized by adding 100 μl of 0.04 N HCl in isopropanol and the viable cell number was estimated as absorbance at 570 nm with a reference at 630 nm.

Statistical analysis. Results are expressed as means ± SE. Statistical significance of differences between mean values was assessed with Student's t-test.

RESULTS

Effect of piroxicam on restitution stimulated by EGF, TGF-β, HGF, and 10% FCS. As shown in Fig. 1, IEC-6 cells migrated considerably faster than Caco-2 cells; IEC-6 cells migrated at a speed of 128.1 ± 17.6 μm/24 h, and Caco-2 cells migrated at the speed of 38.1 ± 3.8 μm/24 h without stimulants. EGF, TGF-β, HGF, and 10% FCS accelerated epithelial restitution by IEC-6 and Caco-2 cells. Each stimulant accelerated epithelial restitution in a dose-dependent manner (data not shown). With IEC-6 cells, maximal effects were observed at 128.3 ± 5.2% of basal resealing speed (resealing speed in the basal medium) in 40 ng/ml EGF, 158.0 ± 5.8% of basal in 10 ng/ml of TGF-β, 177.6 ± 6.6% of basal in 10 ng/ml of HGF, and 257 [illegible] basal in 10% FCS. With Caco-2 cells, maxim[illegible] were observed at 158.8 ± 8.7% of basal resealin[illegible] in 10 ng/ml EGF, 147.8 ± 5.6% of basal in 10 [illegible] TGF-β, 168.3 ± 6.1% of basal in 10 ng/ml H[illegible] 248.3 ± 14.1% of basal in 10% FCS (mean[illegible] n = 6).

To examine the effect of inhibition of endo[illegible] prostaglandin (PG) synthesis, 5 μM piroxica[illegible] added. Piroxicam significantly attenuated the acc[illegible] ing effect of the stimulating factors. The attenuation [illegible] resealing induced by piroxicam seemed almost simil[illegible] in degree between these stimulants. Piroxicam did [illegible] affect the basal resealing rates of IEC-6 and C[illegible] cells. Thus piroxicam appeared to inhibit only [illegible] accelerating effect of these stimulants without aff[illegible] basal resealing. FCS was the strongest of the stimu[illegible] lants we examined for resealing. Therefore, the effect [illegible] piroxicam was further examined in 10% FCS. As [illegible] in Fig. 2, piroxicam slowed the resealing stimulated [illegible] 10% FCS in a dose-dependent manner in both IEC[illegible] and Caco-2 cells. A maximal effect was obtained w[illegible] cells were treated with 5 μM piroxicam.

Secretion of 6-keto-$PGF_{1\alpha}$ by IEC-6 cells and C[illegible] cells. As a representative of PG synthesized by bo[illegible] cells, the amount of PGI_2 secreted into the medium [illegible] 24 h was determined by measuring a stable degrad[illegible] tion product of PGI_2, 6-keto-$PGF_{1\alpha}$, by ELISA[illegible] shown in Fig. 3, IEC-6 cells produced much [illegible] 6-keto-$PGF_{1\alpha}$ than Caco-2 cells, when these cells [illegible] incubated in the basal medium. FCS (10%) enh[illegible] production of 6-keto-$PGF_{1\alpha}$ by ~6.6-fold in IEC-6 [illegible] and 4.1-fold in Caco-2 cells. Inhibition of FCS-stimu[illegible] lated 6-keto-$PGF_{1\alpha}$ production by piroxicam wa[illegible] served in a dose-dependent manner and paralleled with the inhibition of restitution of both cells.

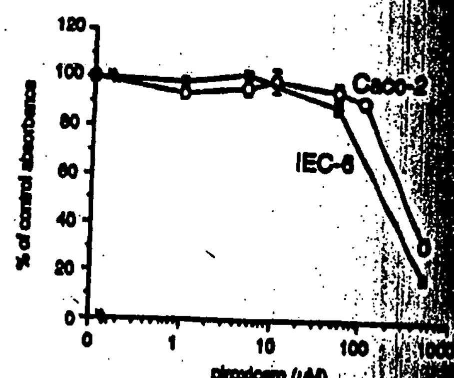

Fig. 4. Effect of piroxicam on viability of IEC-6 (●) and Ca[illegible] cells. Cells were seeded on 96-well culture plates and incubated [illegible] fresh medium under an atmosphere of 5% CO_2-95% air at 37°C [illegible] h. Medium was then changed to fresh medium containing diff[illegible] amounts of piroxicam, and cells were further incubated. Aft[illegible] MTT was added to each well at 1 mg/ml and incubated [illegible] Formazan produced was solubilized by acid isopropanol, and [illegible] cell number was estimated as absorbance at 570 nm with a ref[illegible] at 630 nm. Data are expressed as ratio of control wells. Res[illegible] expressed as means ± SE (n = 4).

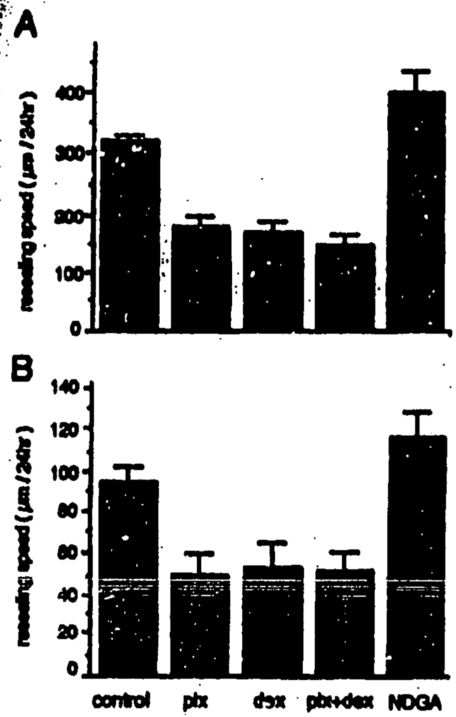

Fig. 5. Effect of piroxicam (pix), dexamethasone (dex), and nordihydroguaiaretic acid (NDGA) on restitution of IEC-6 (*A*) and Caco-2 (*B*) cells. Cells were exposed to MMC for 2 h to inhibit mitosis. Medium was changed to serum-deprived medium 6 h before wounding. Each reagent was added 4 h before wounding. Wound was created, and medium was changed to fresh medium supplemented with each reagent again. Cells were further incubated for 24 h, and resealing speed was determined. Results are expressed as means ± SE (n = 6).

Effect of piroxicam on cell viability. Nonsteroidal anti-inflammatory drugs (NSAIDs), including piroxicam, are supposed to be toxic to intestinal epithelial cells above certain concentrations by uncoupling oxidative phosphorylation and degrading mitochondrial activity (2). Therefore, we examined the effect of piroxicam on cell viability by MTT assay, which is used to evaluate cell viability reflecting mitochondrial activity. As shown in Fig. 4, cell viability loss was observed only when 50 μM or more piroxicam was added to IEC-6 cells and 500 μM or more was added to Caco-2 cells. No effect of piroxicam on viable cell number was observed below these concentrations.

Effect of dexamethasone and NDGA on the epithelial restitution stimulated by 10% FCS. To validate the significance of the cyclooxygenase pathway in the regulation of epithelial restitution, we examined the effect of the synthetic glucocorticoid, dexamethasone, and NDGA on epithelial restitution. Dexamethasone inhibits both the cyclooxygenase and lipoxygenase pathways of arachidonic acid metabolism by inhibiting phospholipase A_2 activity. NDGA inhibits only the lipoxygenase pathway. The FCS-induced increase in resealing by IEC-6 and Caco-2 cells was significantly reduced in a dose-dependent manner by dexame[illegible] shown) with a submaximal inhibition of [illegible] nM dexamethasone in IEC-6 cells and of 50[illegible] dexamethasone in Caco-2 cells. As shown [illegible] inhibition by dexamethasone was almost [illegible] level as that by piroxicam, and these dru[illegible] additive effect on resealing. On the other [illegible] lipoxygenase inhibitor, NDGA, had no effect [illegible] ing by either cell type. Therefore, cyclooxyg[illegible] tabolites, but not lipoxygenase metabolites [illegible] mediate stimulation by FCS of epithelial res[illegible]

Effect of a PGI_2 analogue, OP-41483, [illegible] *restitution.* To examine whether piroxicam[illegible] hibition of epithelial restitution could be com[illegible] by the addition of exogenous PG, the effect [illegible] PGI_2 analogue, OP-41483, which is a compo[illegible] ported to exert strong antiplatelet and antith[illegible] activities (12), was studied. As shown in Fig. 6 [illegible] OP-41483 accelerated the epithelial resealing [illegible] delayed submaximally by 5 μM piroxicam in both I[illegible] and Caco-2 cells. In IEC-6 cells, the effect of OP[illegible]

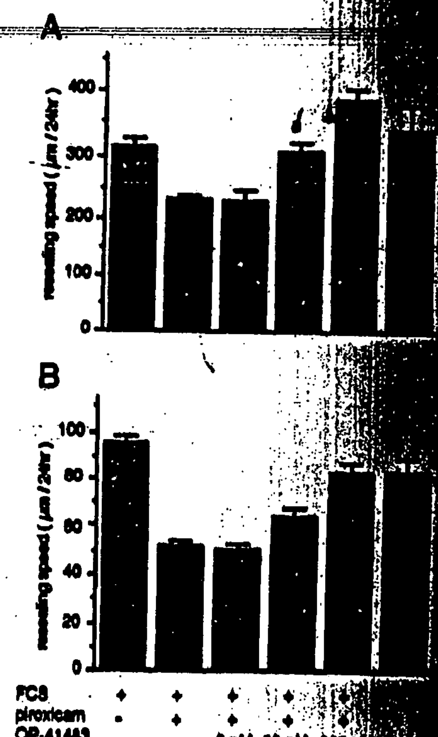

Fig. 6. Effect of a stable prostacyclin analogue, OP-4[illegible] tion by IEC-6 (*A*) and Caco-2 (*B*) cells. Cells were exposed [illegible] 2 h to inhibit mitosis. Medium was changed to [illegible] medium 6 h before wounding. Piroxicam (5 μM) was added [illegible] wounding. Wound was created, and medium was change[illegible] medium supplemented with 10% FCS and 5 μM piroxic[illegible] each concentration of OP-41483. Cells were further incu[illegible] h, and resealing speed was determined. Results are [illegible] means ± SE (n = 6).

was particularly marked, and resealing was accelerated to a rate even faster than control.

DISCUSSION

In the present study, we demonstrated that the accelerating effect of FCS and growth factors on epithelial restitution was partially mediated by endogenous PG. This conclusion was based on the following observations in IEC-6 and Caco-2 cells: *1)* EGF, TGF-β, HGF, and FCS accelerated epithelial restitution, and the acceleration induced by these factors was attenuated by piroxicam; *2)* dexamethasone inhibited the effect of FCS on epithelial restitution almost as much as piroxicam, and no additive effect of piroxicam and dexamethasone was observed; *3)* piroxicam inhibited PG secretion stimulated by FCS at the concentration that submaximally inhibited restitution; *4)* an exogenously added stable PGI_2 analogue, OP-41483, restored the rate of epithelial restitution that was reduced by piroxicam treatment.

This study showed that piroxicam attenuated the accelerating effects of EGF, TGF-β, HGF, and FCS on epithelial restitution. The maximal attenuation was ~50–60% of the stimulated restitution; thus the role of endogenous PG in accelerating epithelial restitution was important, though not exclusive. The signal to stimulate epithelial restitution thus seems to be composed of PG-dependent and -independent pathways. Recently a mitogen-inducible cyclooxygenase gene has been isolated and is getting high attention about the regulation of endogenous PG synthesis and the role in mitogenic signals (8). Neither mitogenic signals nor restitution-accelerating signals are clear. However, the supposed relationship between endogenous PG and mitogenic signals seems like the relationship between endogenous PG and restitution accelerating signals that we observed.

It is described in A-431 cells that both lipoxygenase products and cyclooxygenase products mediated morphological changes evoked by EGF by affecting actin reorganization (19). Such a morphological change may be suitable for faster cell migration. In the present study, we have shown that the restitution accelerating signal was partly dependent on cyclooxygenase products. However, no apparent effect was observed by the lipoxygenase inhibitor NDGA. Therefore, the contribution of lipoxygenase products in our system seemed very little.

The mechanism by which NSAIDs cause specific damage to enterocytes is supposed to be mediated by direct mitochondrial damage during absorption by uncoupling oxidative phosphorylation (2). We have assured by MTT assay that the concentration of piroxicam used in our experiment to inhibit restitution submaximally was far below the level that damages mitochondrial activity. Furthermore, dexamethasone mimicked the effect of piroxicam in inhibiting restitution, and no additive effect of dexamethasone and piroxicam was observed. Therefore, the inhibition of restitution observed in our study was not due to toxicity of piroxicam.

It has been reported that PG production is increased in the inflamed mucosa of inflammatory bowel disease and these metabolites were previously thought to promote the inflammatory process in the intestinal mucosa (9). However, unlike lipoxygenase products, PG have recently been reported to mediate protective effects in the intestinal epithelium. For example, NSAIDs, which inhibit PG synthesis, sometimes trigger the recurrence of quiescent inflammatory bowel disease (21). Bjarnason et al. (3) have reported that >60% of patients on long-term NSAID therapy may have an asymptomatic enteropathy. Moreover, NSAIDs have been reported to cause colitis in experimental animals only when irritants, such as bacteria, are present in the lumen (22). Our observation that endogenous PG mediates the acceleration of epithelial restitution by growth factors may be one mechanism underlying the protective effects of endogenous PG in the intestinal epithelium.

In summary, we have demonstrated that intestinal epithelial restitution was modulated by endogenous PG. The finding that endogenous PG promotes intestinal epithelial restitution may become a clue to finding an appropriate pharmacological approach to stimulating the restitution process to treat diseases presumably related to epithelial barrier function, such as inflammatory bowel disease.

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CERTIFICATE

I, the undersigned, a Registered Patent Attorney, do hereby certify that the attached English translation was translated into English faithfully and accurately from Japanese Patent Application Laid-open No. 8-231418, Laid-open date September, 10. 1996.

Dated this 19th day of October 1998

By ____________________
Yukihiro Ikeda
Patent Attorney

Japanese Patent Application Laid-Open No. 8-231418

【Title of the Invention】 Therapeutic Agent for Intestinal Disease

【Abstract】

【Constitution】 A preventive · therapeutic agent for intestinal diseases which comprises as an active ingredient a hepatic parenchyma cell growth factor (for example, 1) having a molecular weight of about 76 to 92 K when it is estimated by the SDS-PAGE; 2) having an activity to grow hepatic parenchyma cells; 3) losing said activity by the heat treatment at 80℃ for 10 minutes; 4) also losing said activity by the digestive treatment with trypsin and the digestive treatment with chymotrypsin; and 5) having a high affinity with heparin.

【Effects】 The active ingredient of the present pharmaceutical agent, HGF, exhibits an action to promote the cellular growth and an action to generate the organs in the cells derived from the intestine and is useful to the prevention · therapy of intestinal diseases such as inflammatory colitis.

【Scope of Claim for a Patent】

【Claim 1】 A preventive · therapeutic agent for intestinal diseases, comprising a hepatic parenchyma cell growth factor as an active ingredient.

【Claim 2】 A preventive · therapeutic agent according to Claim 1, wherein the hepatic parenchyma cell growth factor shows the following physico-chemical properties:

1) having a putative molecular weight of about 76,000 to 92,000 estimated by SDS-PAGE (under non-reducing conditions);

2) exhibiting an activity to grow the hepatic parenchyma cells;

3) losing said activity by a heat treatment at 80℃ for 10 minutes;

4) also losing said activity by a digestive treatment with trypsin and a digestive treatment with chymotrypsin; and

5) having a high affinity with heparin.

【Claim 3】 A preventive · therapeutic agent according to Claim 1 or 2, wherein the intestinal diseases are an inflammatory disease in a lower digestive tract.

【Detailed Description of the Invention】

【0001】

【Technical Field to which the Invention Pertains】 The present invention relates to a preventive · therapeutic agent for intestinal diseases. More particularly, it relates to a pharmaceutical agent containing a hepatic parenchyma cell growth factor as an active ingredient, useful in the prevention and/or therapy of the intestinal diseases such as inflammatory intestinal disease.

【0002】

【Prior Art】 The intestinal diseases may sometimes be accompanied by a tissue anomaly such as ulcer or inflammation in the epithelium of intestinal tissues. It has been known that those diseases are typically represented by an inflammatory intestinal disease giving rise to a disorder chiefly on the mucous membranes or in the substratum of the mucous membranes in the intestine. The name of those diseases is used to mean many things comprehensively. They include an ulcerous colitis and a Crohn's disease, and intestinal diseases due to bacterial, parasite or viral infection, and intestinal diseases due to radiation ray, pharmaceutical agent or chemical substance, and an ischemic

colitis, a Behcet's disease, an isolated rectum ulcer and the like. (Pathology & Clinic, 10, pp. 142-151, 1992). Of those diseases, there were not much more than about 30,000 patients with ulcerous colitis or Crohn's disease in 1990, but the diseases are a big clinical problem because they are intractable in comparatively many cases and the number of the involved patients is increasing rapidly. Besides the surgery such as excision of inflammation sites, the ulcerous colitis and Crohn's diseases are treated by the trial administration of a salazoupyrine, a metronidazole, a steroid or an immunosuppressive agent. However, the cause for those diseases has not been made clear as yet, and it cannot be said that good pharmacotherapies are available for them (Progress of Medicine, 160, pp.895-898, 1992).

【0003】 On the other hand, a human hepatic parenchyma cell growth factor (hereinafter referred to as "hHGF", and a hepatic parenchyma cell growth factor in general referred to as "HGF") was separated from the plasma of the fulminating hepatitis patients for the first time. The so obtained human hepatic parenchyma cell growth factor served as a protein factor of human origin to promote the growth of the hepatic cells of primary culture (Japanese Patent Application Laid-Open No. 63-22526). Thereafter, a gene encoding hHGF protein (cDNA), an amino acid sequence thereof (Japanese Patent Application Laid-Open No. 3-72883) and a production method of recombinant hHGF (Japanese Patent Application Laid-Open No.3-285693) have been reported. Those recombinant hHGFs (hereinafter referred to as "rhHGF") can act as a promoter of the growth and function of the hepatic parenchyma cells in vitro (J. Clin. Invest., 87, pp. 1853-1857,

1991) and in vivo (Jpn. J. Pharmacol., 59, suppl. 1, 137, 1992).

【0004】 Furthermore, the target cells or target tissues of the HGF have been investigated extensively. It has been reported that, besides the hepatic cells, the HGF reacts with various epithelial cells (tubular epithelium, lung epithelium, bile-duct epithelium or stomach epithelium and the like), fibroblasts, lymphoid cells and the like, resulting in changes in their growth or motility. (Mitsubishi Kasei R&D Review, 7, pp. 16-24, 1993). It has also been established that products of the carcinogenic gene c-met work on the HGF-target cells as a receptor molecule. (Science, 251, pp. 802-804, 1991).

【0005】 Of manifold biological activities of the HGF, that of improving the cellular dispersion of epithelial cells in a petri dish has conventionally been designated as a scatter factor (SF: motility-improving activity). (Nature, 327, pp. 239-242, 1987). Later, a cell strain derived from the kidney and exhibiting a motility-improving activity in response to the HGF has been found to form a lumen depending upon the HGF when the cell strain is cultured in collagen gel. As a result, it has been conceived that the changes in motility on account of the HGF are not limited to the mere improvement of cellular motility but they are one step of the genesis (regenesis) of organs. (Cell, 67, pp.901-908, 1991). However, there have been no reports on biological activity of the HGF on the intestinal tissues and the cells of intestinal origin and particularly on their growth and motility.

【0006】

【Problems to be Solved by the Invention and Means for Solving the Problems】 An object of the present invention is to provide a

preventive · therapeutic agent for intestinal diseases. More particularly, it is to provide a pharmaceutical agent useful in the prevention · therapy of various intestinal diseases including an ulcerous colitis, Crohn's disease and the like. The present inventor has tried to find a solution in said problem in every way. As a result, it has been found that the HGF can promote the growth of cells derived from the intestine and the genesis (regenesis) of their organs and is useful in the prevention and/or therapy of the various intestinal diseases such as inflammatory intestinal diseases for which good therapies have not been established as yet. The present invention has been completed on the basis of those findings.

[0007] Therefore, the present invention provides a preventive · therapeutic agent for intestinal diseases, which comprises a hepatic parenchyma cell growth factor as an active ingredient. According to a preferable aspect of the present invention, there is provided preventive · therapeutic agent wherein the hepatic parenchyma cell growth factor shows the following physicochemical properties: 1) having a putative molecular weight of about 76,000 to 92,000 when it is estimated by SDS-PAGE (under non-reducing conditions); 2) having an activity of growing the hepatic parenchyma cells; 3) losing said activity by a heat treatment at 80℃ for 10 minutes; 4) also losing said activity by a digestive treatment with trypsin and a digestive treatment with chymotrypsin; and 5) having a high affinity with heparin, as well as the preventive · therapeutic agent wherein the intestinal diseases are an inflammatory disease in a lower digestive tract. Another aspect of the present invention is a cellular growth-promoting

agent and／or organogenesis-promoting agent for the cells of intestinal origin, which comprises a hepatic parenchyma cell growth factor or said preferable hepatic parenchyma cell growth factor as an active ingredient.

【0008】 The pharmaceutical agents of the present invention are characterized by comprising a hepatic parenchyma cell growth factor (HGF) as an active ingredient. The present invention can use the hepatic parenchyma cell growth factors isolated and purified from the humors or tissues derived from the mammal such as human or rat that are known as containing the HGF or from the cells capable of producing the HGF voluntarily. The present invention also can use recombinant HGFs obtained by the gene recombination introducing cDNA of the HGF into cells. Preferably, the HGFs of human origin (hHGF) are used as the active ingredient in the pharmaceutical agents of the present invention.

【0009】 Hosts to be used for producing the recombinant HGF are not particularly limited but, for example, E. coli, Bacilli subtilis, yeast, filamentous fungi, plant cells, insect cells, animal cells and the like may be suitably used. More specifically, the examples of the host cells are placentas derived from said mammal, tissues or blood of the patients with hepatic disease and fibroblast strains such as MRC-5 cells and IMR-9 cells, preferably CHO cells, and the like. HGF-producible transformants can be manufactured by incorporating expression vectors that contain cDNA encoding the HGF, preferably hHGF into those host cells in accordance with, for example, the method described in Japanese Patent Application Laid-Open No. 3-285693. The so obtained transformants are

cultured, and the HGFs are separated and collected therefrom. Such HGFs are preferably used in still another aspect of the present invention.

【0010】 The pharmaceutical agents of the present invention may contain a non-natural HGF as the active ingredient in place of said natural or recombinant HGF per se. The non-natural HGFs are obtained by altering the natural HGFs with the substitution, deletion, insertion, modification and the like of parts of amino acid, as much as precursor protein of the natural HGFs or their activity of growing the hepatic parenchyma cells are held undamaged. As the non-natural HGFs, the present invention can use those described in Japanese Patent Application Laid-Open No. 2-288899, PCT International Publication WO90／10651, Japanese Patent Application Laid-Open No. 3-130091, ditto No. 3-255096 and ditto No. 4-30000 and in the publications such as Nature, 342, pp. 440-443, 1989.

【0011】 In the pharmaceutical agents of the present invention, the active ingredient is more preferably the HGFs with the following physicochemical properties:

1) having a putative molecular weight of 76,000 to 92,000 estimated by SDS-PAGE (under non-reducing conditions);

2) having an activity to grow the hepatic parenchyma cells;

3) losing said activity by a heat treatment at 80℃ for 10 minutes;

4) also losing said activity by a digestive treatment with trypsin and a digestive treatment with chymotrypsin; and

5) having a high affinity with heparin.

Of this kind of HGFs, those of human origin are preferable. Particularly, the hHGFs identified by an amino acid sequence of

Japanese Patent Application Laid-Open No. 3-72883 or Japanese Patent Application Laid-Open No. 4-89499 are more preferable.

【0012】 The pharmaceutical agents of the present invention are prepared from one or more kinds of said HGFs singly or in combination with appropriate pharmaceutical additives, making dosage forms of medicinal composition, preferably for parenteral administration. The dosage forms of those medicinal compositions are not particularly limited as long as they are administered parenterally in general and, for example, the medicinal compositions may be filled into ampoules for injection or lyophilized into powder for injection (to be filled into vials). The pharmaceutical agents of the present invention can be prepared in a variety of dosage forms by using well-known pharmaceutical additives such as diluents and adjuncts that are readily available to persons skilled in the art in accordance with the procedures accustomed to them.

【0013】 For example, the pharmaceutical agent in the form of lyophilized powder for injection is manufactured in the following way: an effective amount of said HGF as purified is dissolved in a diluent such as distilled water for injection, physiological saline water or aqueous glucose solution. As occasion demands, the so obtained solution is added with the additives. Those additives include an excipient such as carboxymethylcellulose and sodium alginate; a stabilizer such as polyethylene glycol, sodium dextran sulfate, amino acid and human serum albumin; a preservative such as benzyl alcohol, bezalkonium chloride and phenol; an analgesic agent such as glucose, calcium gluconate and procaine hydrochloride; a pH adjuster such as hydrochloric acid, acetic acid,

citric acid and sodium hydroxide. Then, the mixture is lyophilized by a conventional method to complete desired powder.

【0014】 The pharmaceutical agent to be filled into the ampoules for injection is manufactured in the following way: an effective amount of said HGF is dissolved in a diluent such as distilled water for injection, physiological saline water or Ringer's solution. As occasion demands, the so obtained solution is added with the additives. Those additives include a dissolution assistant such as sodium salicylate and mannitol; a buffer such as sodium citrate and glycerin; an isotonic-making agent such as glucose and invert sugar; said stabilizer; said preservative; said analgesic agent, said pH adjuster and the like. Then, an ordinary heat treatment or sterile filtration to complete the desired pharmaceutical agent sterilizes the mixture. The heat sterilization process sometimes inactivates the active ingredient depending upon its kind and an appropriate sterilizing method should be selected according to the circumstances.

【0015】 Although it is not intended to adhere to any particular theory, the active ingredient, HGF, of the present pharmaceutical agents exhibits an action to promote the cellular growth and the genesis (regenesis) of organs in the cells of intestinal origin. More specifically, the HGF promotes the growth of the intestinal cells by stimulating DNA to synthesize in the cells of intestinal origin. Furthermore, the HGF induces the intestinal cells to generate the organs by exhibiting an action to improve motility (SF) and thus promoting the formation of lumens in the intestinal cells. There have been reports on the cellular growth-promoting action of HGFs in the hepatic cells and reports on the motility-

improving activity and lumen formation-promoting action of the HGF in the cells derived from the kidney. But those on the same actions of HGF in the cells of intestinal origin have not as yet been published. Since the hepatic cells and the cells of renal origin have no bearing on the cells of intestinal origin in terms of embryology and cytology, said actions of HGF on the intestinal cells are a great surprise.

【0016】 The pharmaceutical agents of the present invention are useful to the prevention and／or therapy of the intestinal diseases. The pharmaceutical agents of the present invention can find their application in various intestinal diseases but are preferably used in the prevention and／or therapy of inflammatory intestinal diseases giving rise to disorders on the mucous membranes or in the substratum of mucous membranes in the intestine. Examples of such inflammatory intestinal diseases are an ulcerous colitis and a Crohn's disease, and the intestinal diseases due to bacterial, parasite and viral infections, and the intestinal diseases due to radiation rays, pharmaceutical agents and chemical substances, and an ischemic colitis, a Behcet's syndrome, an isolated ileum ulcer and the like. The pharmaceutical agents of the present invention are indicated preferably for the inflammatory diseases in the lower digestive tract including the small and large intestines and more preferably for inflammatory diseases in the lower digestive tract including the small and large intestines but excluding the duodenum. Most preferably, they are indicated for the inflammatory diseases in the large intestine. The pharmaceutical agents of the present invention are not limited to be indicated for the diseases as

enumerated above. They can be indicated for any diseases receptive of the therapeutic and/or preventive effects resulting from promoting the growth of the intestinal cells and thus speeding up the genesis of organs.

【0017】 The pharmaceutical agents of the present invention may be blended with the active ingredient of other pharmaceutical agents exhibiting a pharmacological action similar or dissimilar to that of the present pharmaceutical agents. It has been known that the sulfated polysaccharides such as heparin and dextran sulfate and their derivatives can induce the active ingredient of the present pharmaceutical agents to increase its action to promote the growth of the hepatic parenchyma cells (Japanese Patent Appli-cation Laid-Open No. 5-301824). Therefore, the sulfated polysaccharides and their derivatives may as well be blended into the active ingredient of the present pharmaceutical agents. Since those polysaccharides and their derivative exhibit an action to improve stability of the present pharmaceutical agents, their blending is still another preferable aspect of the present invention.

【0018】 Generally, the pharmaceutical agents of the present invention are administered parenterally and, more specifically, subcutaneously, intramuscularly or intravenously for the purpose of the prevention and/or therapy of said diseases in mammal including human being. A predetermined amount of the present pharmaceutical agents is administered generally by injection in one single dose or in a plurality of doses or continuously administered by instillation and the like. The dosage should be determined, depending upon the age, sex, conditions and

bodyweight of the patients and the route of administration and the like. In general, an adult patient suitably receives the present pharmaceutical agents in a range of 1 μg/kg to 10 mg/kg, preferably 10 to 1000 μg/kg per day.

【0019】

【Examples】 The present invention will be described in greater detail below, with reference to examples, but those examples should not be construed as limiting the scope of the present invention. In those examples, a recombinant hHGF (rhHGF) manufactured by the method described in Japanese Patent Application Laid-Open No. 3-285693 was used as the HGF. Furthermore, it had previously been observed that, when the cell strains were cultured under the ordinary conditions by using the fetal bovine serum (FBS) in a concentration of about 5 to 10%, they were grown with too much dependence on the FBS. Therefore, the cell strains capable of growing in a low FBS concentration of 0 or not much more than 1% and detecting the reactivity with HGF were put to use. Three kinds of the cell strains of intestinal origin were obtained from Dainippon Pharmaceutical Co., Ltd. and used in the following experiments. The so obtained cell strains included (1) Intestine 407: derived from the human embryonoid small intestine; (2) IEC-6: an epithelial cell derived from the rat small intestine; and (3) IEC-18: an epithelial cell derived from the rat ileum.

【0020】 Example 1: The action of HGF on the DNA synthesis in the cells of intestinal origin

Said cell strains were grown to the semi-confluence, exfoliated with a trypsin solution (Sigma Corporation) and

suspended again in an Eagle's MEM medium containing 0.25% of fetal bovine serum. The number of cells in the suspension was counted, and the suspension was inoculated on 96 well plates respectively in an amount of 100 μl, to make 10^4 cells per well. The cells hardly to grow without serum were added with the bovine serum to make the final bovine serum concentration of 1%. Then, a suspension of hHGF in 0.25% of bovine serum albumin (BSA) was added to the cell-inoculated 96 well plates respectively in an amount of 100 μl. The mixture was adjusted to make the final HGF concentration of 0 to 1,000 ng/ml, and the plates were incubated in a CO_2 incubator (5% CO_2-95% air) for 24 hours. In final 4 hours of the culture, the cells were labeled with ^3H thymidine in an amount of 1 μCi/well and thereafter collected on a glass filter by a cell harvester (Pharmacia Corporation). The cells were well washed and dried and a scintillator was added to them. Thereafter, the radioactive activity incorporated into the cells was measured by a liquid scintillation counter (Hewlett-Packard Corporation).

【0021】 Fig. 1 and Fig. 2 are diagrams showing the action of hHGF to promote the synthesis of DNA with dependence on the hHGF dose in the cells of intestinal origin. The concentration of HGF (ng/ml) was plotted in terms of logarithm on the axis of abscissa. The uptake of ^3H thymidine (cpm) by cellular DNA was plotted as an index of cell growth on the axis of ordinates. In Fig. 1, ○ represents the uptake of thymidine by Intestine 407 grown under the condition of no serum and ● the uptake of thymidine by IEC-6 grown under the condition of 1% of serum. In Fig. 2, ○ represents the uptake of thymidine by IEC-18 grown under the

condition of no serum and ● the uptake of thymidine by IEC-6 grown under the condition of no serum.

【0022】 It is evident in all the cases that the growth of the cells of intestinal origin was promoted, depending upon the HGF concentration. On the other hand, it was found that the cell growth had a tendency of inhibition in a range of high HFG concentrations (＞20 ng／ml). The same findings had previously been reported as a result of a study using the hepatic cells. The detail of the involved mechanism has remained unknown now. But the mechanism would presumably be accounted for by the fact that the peak occurs in the derived DNA synthesis after 48 hours in the presence of high HGF concentrations, although it is not intended to adhere to any particular theory. (Exp. Cell Res., 209, pp. 317 to 314, 1993).

【0023】 Example 2: The action of hHGF to improve motility or promote the genesis of organs in the cells of intestinal origin

It is evident from the results as described above that the HGF had the activity to promote the growth of the cells of intestinal origin in all the cases wherein 3 cell strains of intestinal origin were tested. Thus, an investigation was conducted in an attempt to make clear the action of hHGF on motility and the genesis of organs in the cells of intestinal origin. In the investigation, a predetermined amount of hHGF was added to a system culturing the cells of intestinal origin in the presence of 10% or 0.5% of FBS, comparing the state of cells in the HGF-added system with that of the HGF-free system after 24 hours.

【0024】 The 3 cell strains of intestinal origin used in Example 1 were exfoliated with the trypsin solution and suspended again in

an Eagle's MEM medium containing 10 or 0.5% of FBS, to make 0.625 to 5×10^4 cells／ml. The cell suspension was inoculated on 24 well plates (Coster Corporation) respectively in an amount of 1 ml. Immediately thereafter, the hHGF or hHGF-suspended buffer (the Eagle's MEM medium containing 0.25% of BSA) in the final concentration of 40 ng／ml was added to the cell suspension and the two were mixed and the plates were incubated in the CO_2 incubator (5% CO_2-95% air) for 24 hours. Then, the cells were observed by an inverted microscope (NIKON CORP.: 100 to 200 magnifications) and photographed by a Polaroid camera (Polaroid Corporation).

【0025】 Fig. 3 shows the results of the investigation that the hHGF having a physiological concentration (40 ng／ml) can exhibit the action to improve motility in the cell strains of intestinal origin, IEC-6 (a, b) and IEC-18 (c, d). (b and d show the results in the presence of hHGF). Furthermore, when the hHGF (40 ng／ml) acted on the cell strain of intestinal origin, IEC-18, in the presence of 0.5% of FBS, a stereostructure in the shape of a lumen was observed in the cells of intestinal origin, evidencing that the genesis of the organ was promoted. (Fig. 4, a shows the state of cells before the hHGF was added and b shows the state of cells after the hHGF was added).

【Effects of the Invention】 The active ingredient of the present pharmaceutical agents can exhibit the action of growing the cells, the action of improving motility and the action of generating the organs in the cells of intestinal origin. Therefore, the present pharmaceutical agents are useful to the prevention and／or therapy of the intestinal diseases such as ulcerous colitis and

inflammatory colitis.

【Brief Description of the Drawings】

【Fig. 1】 is a diagram showing the action of hHGF to promote the synthesis of DNA with dependence on the hHGF dose in the cells of intestinal origin. In the figure, the axis of abscissa plots the concentrations of HGF (ng／ml) in terms of logarithm and the axis of ordinates plots the uptake of ^{3}H-thymidine (cpm) by cellular DNA as an index of the cell growth.

○ represents the results of Intestine 407 and ● those of IEC-6.

【Fig. 2】 is a diagram showing the activity of hHGF to promote the synthesis of DNA with dependence on the hHGF dose in the cells of intestinal origin. In the figure, the axis of abscissa plots the concentrations of HGF (ng／ml) in terms of logarithm and the axis of ordinates plots the uptake of ^{3}H-thymidine (cpm) by cellular DNA as an index of the cell growth. ○ represents the results of IEC-18 and ● those of IEC-6.

【Fig. 3】 is a photograph in place of a diagram, to show the state of cells (biological) accounting for the action of hHGF to improve motility in the cells of intestinal origin. a and b are for IEC-6 and c and d are for IEC-18. a and c represent the results before and b and d those after the hHGF was added.

【Fig. 4】 is a photograph in place of a diagram, to show the state of cells (biological) accounting for the action of hHGF to promote the genesis of the organs in the cells of intestinal origin. a represents the results before and b those after the hHGF was added.

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Hepatocyte Growth Factor Region Specifically Stimulates Gastro-Intestinal Epithelial Growth in Primary Culture

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Summary: Hepatocyte growth factor (HGF) stimulated the growth of fetal rat gastro-intestinal epithelial cells in primary culture with a clear dose-response relationship. The epithelial response to the mitogenic activity of HGF was different among the region of the gastro-intestinal tract; glandular stomach most responsive, followed by intestine and forestomach. The interaction of HGF with other growth factors in inducing the epithelial growth was also different depending on the type of the epithelial cells, indicating a region-specific growth regulation in the gastro-intestinal tract. Analyses using Northern blot and RT-PCR revealed that HGF mRNA was expressed only in mesenchymes but not in epithelia of the gastro-intestinal tract while expression of *c-met* (HGF receptor) gene was observed in both tissues. These results suggest that gastro-intestinal mesenchymes secrete HGF which stimulates the growth of attaching epithelial cells by a paracrine mechanism, and that the epithelial response to HGF is controlled by a region-specific growth regulatory mechanism.

Introduction: Hepatocyte growth factor (HGF) was first purified from the plasma of partially hepatectomized animals on the basis that it stimulated DNA synthesis of cultured hepatocytes (1, 2, 3). Recent studies show that HGF stimulates growth of not only hepatocytes but also various epithelial cells (4). Takahashi et al. (5) recently reported that HGF stimulated growth of rabbit gastric epithelial cells in primary culture, but they stated that significant fraction of their cultures was fibroblastic cells. Thus it remains to be examined whether HGF stimulates gastric epithelial growth directly or via contaminated fibroblastic cells. Also it is of interest whether HGF affects epithelial

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growth of other gastro-intestinal organs. We have reported primary culture systems for intestinal (6), glandular stomach (7) and forestomach epithelial cells (8). In our systems, pure epithelial cells without any contamination of fibroblastic cells could be obtained from various parts of gastro-intestinal tract, and growth factor dependency of the epithelial cells has been clearly defined (8). Here we examined effects of HGF on the growth of various gastro-intestinal epithelial cells in primary culture.

Materials and Methods

Hepatocyte growth factor: Recombinant human HGF was kindly supplied by Dr. Takehisa Ishii of Research Center, Mitsubishi Kasei Corp., Japan.
Cell culture: Forestomach, glandular stomach and intestinal epithelial cells were obtained from 16.5-day fetuses of Fischer rats (Charles River Japan), and cultured as previously reported (8). Intestinal epithelial cells were cultured in F12 medium supplemented with bovine serum albumin (1 mg/ml), epidermal growth factor (EGF; 20 ng/ml), insulin (30 μg/ml), cholera toxin (0.2 μg/ml), transferrin (100 μg/ml) and hydrocortisone (2 μg/ml). Forestomach and glandular stomach epithelial cells were cultured in F12 medium supplemented with horse serum (10%), bovine pituitary extract (100 μg/ml), EGF (10 ng/ml), insulin (3 μg/ml), cholera toxin (0.3 μg/ml) and hydrocortisone (3 μg/ml). The cells were seeded on collagen gels and incubated in a humidified atmosphere of 5% CO_2 in air at 37°C. The epithelial growth was examined on day 4 in culture as has been described (6). To reduce variation between experiments, cell numbers were expressed as a percentage by regarding the cell number in standard culture condition as 100%. The data were analyzed by the Student's *t*-test, and differences were considered significant when $p<0.05$.
RNA extraction and Northern hybridization: The epithelial and mesenchymal tissues of forestomach, glandular stomach and duodenum were obtained from 16.5-day rat fetuses, and total RNA was extracted from the tissues by the guanidine isothiocyanate method (9). A sample of 2 μg of poly(A^+) RNA was electrophoresed and analyzed by Northern blotting as described elsewhere (10). Rat HGF and *c-met* cDNA fragments were prepared by polymerase-chain-reaction (PCR) amplification as described below, labeled with [α-P^{32}]dCTP using random-primer labeling kits (Amersham, UK), and used as the probe for hybridization.
Reverse transcription-polymerase chain reaction (RT-PCR): Primers specific for the amplification of rat HGF gene (5'-primer, 5'-CCAACACAAACAACAGTAGG-3'; 3'-primer, 5'-AACAATGACACCAAGAACCA-3'; size of the amplified fragment, 585 bp), those for *c-met* gene (5'-primer, 5'-TGATGAATGCCCCAGCGGTA-3'; 3'-primer, 5'-TTCATTGCCCATTGAGAT-3'; size of the amplified fragment, 905 bp) and those for β-actin gene (5'-primer, 5'-ATCGTGGGCCGCCCTAGGCA-3'; 3'-primer, 5'-TGGCCTT-AGGGTTCAGAGGGG-3'; size of the amplified fragment, 244 bp) were synthesized with a DNA synthesizer (Applied Biosystems, USA). For each reaction, 1 μg of poly(A^+) RNAs from respective tissues was reverse transcribed to cDNAs using cDNA synthesis kit (Pharmacia LKB, Sweden), and the resulting cDNAs were subjected to 25 to 40 cycles of PCR using AmpliTaq DNA polymerase (Perkin Elmer Cetus, USA). The PCR products were electrophoresed through a 2.0% agarose gel in 1xTBE buffer.
For the quantification of HGF and *c-met* mRNA levels, competitive PCR method was used according to Gilliland et al.(11). As competitive templates, subcloned DNA fragments were prepared from HGF or *c-met* cDNAs by introducing small deletions. Equal amounts of cDNA products were amplified in the presence of serial dilutions of

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Fibroblasts and transforming growth factor beta induce organization and differentiation of T84 human epithelial cells.

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BACKGROUND & AIMS: The gut epithelium in the crypt-villus axis represents a continuous developmental system in which the role of fibroblast epithelial interactions is obvious. The aim of this study was to establish an in vitro method whereby fibroblast-guided differentiation of crypt-like gut epithelial cells can be studied. METHODS: Intestinal epithelial cells (T84 an HT-29) were cultured within type I collagen gel together were fibroblasts without cell-to-cell contact. T84 cells were also grown in the presence of transforming growth factor beta and hepatocyte growth factor. The gels were studied using light and electron microscopy and histochemical and immunohistochemical methods. RESULTS: The epithelial cells formed unorganized cell clusters within the gels, but when given fibroblast support, 76% of the T84 cell colonies (not HT-29) organized into luminal formations, and basement membranes including laminin were well deposited. The cells i the columnar single cell-layer luminal formations (49% of all colonies) were differentiated, showing microvilli, up-regulated alkaline phosphatase brush border activity, and mucin profiles typical for small intestine. This fibroblast- induced organization and differentiation was induced by transforming growtl factor beta. CONCLUSIONS: Crypt-like T84 epithelial cells are able to differentiate when grown three-dimensionally together with fibroblasts or transforming growth factor beta. This method may be used for mesenchymal- epithelial cell cross-talk studies.

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Fibroblasts and Transforming Growth Factor β Induce Organization and Differentiation of T84 Human Epithelial Cells

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Background & Aims: The gut epithelium in the crypt-villus axis represents a continuous developmental system in which the role of fibroblast-epithelial interactions is obvious. The aim of this study was to establish an in vitro method whereby fibroblast-guided differentiation of crypt-like gut epithelial cells can be studied. *Methods:* Intestinal epithelial cells (T84 and HT-29) were cultured within type I collagen gel together with fibroblasts without cell-to-cell contact. T84 cells were also grown in the presence of transforming growth factor β and hepatocyte growth factor. The gels were studied using light and electron microscopy and histochemical and immunohistochemical methods. *Results:* The epithelial cells formed unorganized cell clusters within the gels, but when given fibroblast support, 76% of the T84 cell colonies (not HT-29) organized into luminal formations, and basement membranes including laminin were well deposited. The cells in the columnar single cell–layer luminal formations (49% of all colonies) were differentiated, showing microvilli, up-regulated alkaline phosphatase brush border activity, and mucin profiles typical for small intestine. This fibroblast-induced organization and differentiation was induced by transforming growth factor β. *Conclusions:* Crypt-like T84 epithelial cells are able to differentiate when grown three-dimensionally together with fibroblasts or transforming growth factor β. This method may be used for mesenchymal-epithelial cell cross-talk studies.

The small intestinal crypt-villus axis is an interesting model for epithelial cell proliferation and differentiation.[1] Recent studies have shown that mesenchymal-epithelial instructive interactions as well as interactions with extracellular matrix are needed for epithelial cell movement and differentiation.[2,3] Mesenchymal fibroblasts, which lie just beneath the epithelial basement membrane in the intestine,[4] especially play a central role in epithelial cell biology through the growth factors and cytokines they produce.[5–7]

Hepatocyte growth factor (HGF) and transforming growth factor (TGF)-β are known fibroblast-derived factors affecting epithelial cell proliferation, differentiation, and motility (for review, see Matsumoto and Nakamura[8] and Derynck[9]). HGF has recently been shown to be a mitogen, motogen, and morphogen for murine kidney epithelial (MDCK) cells. In fact, HGF was shown to induce the tubulus formation of MDCK cells when grown within collagen gels,[10] and it is now obvious that this fibroblast-derived growth factor is important for epithelial cells in development and organogenesis.[11,12] TGF-β works in the opposite way, inhibiting epithelial cell motility and arresting proliferation, often with simultaneous signaling for the terminal differentiation pathway,[9,13,14] and it seems to work as an antagonist for HGF.[15]

Hayward and Whitehead[16] studied the three-dimensional behavior of a colon carcinoma cell line. However, the role of fibroblasts in three-dimensional gut epithelial cell organization and differentiation has not been reported. As targets for fibroblast-derived growth factors, we chose the commonly used human intestinal epithelial cell lines HT-29 and T84. The cell line T84, derived from a colon carcinoma lung metastase, is widely used as a model for Cl^- secretion studies and crypt cell biology.[17] Although representing transformed cells, the T84 cell model evinces striking similarities to the natural mammalian epithelium in the regulation of Cl^- secretion. T84 cells display the phenotype of the natural crypt epithelium; when grown as confluent monolayers on collagen-coated permeable supports, they form well-lineated intracellular junctions including continuous circumferential tight junctions.[18] These cells are also capable of goblet cell differentiation and mucin secretion.[19] How-

Abbreviations used in this paper: BME, basal medium (Eagle); DMEM/F-12, a nutrient mixture of Dulbecco's modified Eagle medium and Ham's F-12; FCM, fibroblast-conditioned medium; FCS, fetal calf serum; FS, foreskin fibroblast; HGF, hepatocyte growth factor; hHGF, recombinant human HGF; LAP, latency-associated protein; MDCK, murine kidney epithelial cell line; hTGF-β1, recombinant human transforming growth factor β1; TGF, transforming growth factor.

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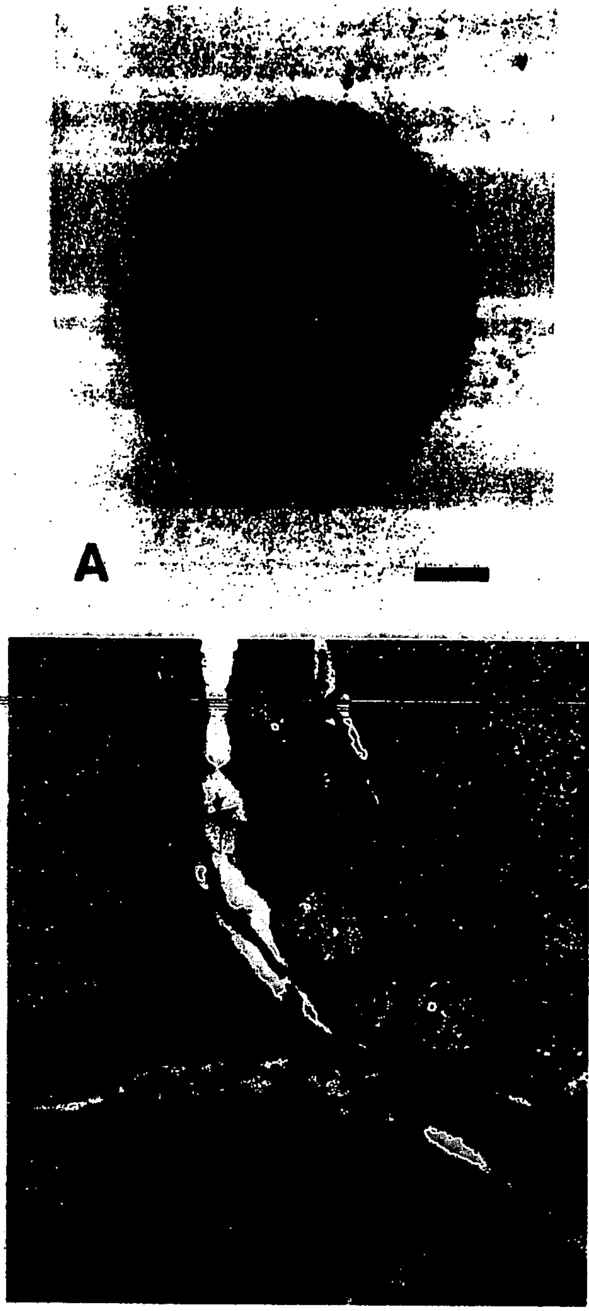


Figure 1. (*A*) Three-dimensional cultures (10 days) of human intestinal epithelial cells (T84) without fibroblast support form round colonies (hematoxylin staining; *bar* = 30 μm). (*B*) Electron microscopy of the colonies shows loosely packed cells with wide intercellular spaces (*arrowheads*) and occasional cell junctions (*arrow*).

dimensional organization of T84 intestinal epithelial cells. In control experiments, organization occurred in only 8% of the colonies (range, 0%–25%; 15 independent experiments). FS-, IMR-90–, and FCM-induced lumenal formation differed significantly from that of controls ($P < 0.001$) (Figure 2).

In light microscopy, two types of T84 one-cell layer luminal formations were noticed (Figure 3). In one type the lumens were large and the mean height of the epithelial cells was 12 μm (SD, 4 μm; n = 58), whereas the other showed a smaller lumen with an average epithelial cell height of 40 μm (SD, 10 μm; n = 63). In the latter, the nuclei of the cells were situated in the basal poles. Under electron microscopy, the thinner epithelial cells were cuboidal with irregular and distorted microvilli (Figure 4*A*). The large lumen of these organized epithelial cell colonies indicates these crypt-like cells to be in a secretory stage. The taller epithelial cells were morphologically columnar, and the microvilli were well developed (Figure 4*B*). The skeleton of the microvilli and the cytoplasmic anchorings were well formed. In both types of luminal formations, goblet cells were occasionally found (data not shown). In the luminal formations, the epithelial cells expressed well-formed apical intercellular junctions and the intercellular spaces were tight.

We hypothesized that the morphologically differentiated columnar type of cells with basal nuclei and microvilli facing the lumen also show functional differentiation. To show this, expression of brush border alkaline phosphatase, mucine profiles, and sucrase isomaltase were studied. Moderate to strong alkaline phosphatase staining was found only at the apical sites of T84 luminal formations. In cocultures with FS, differentiated columnar T84 cells in the luminal formations showed moderate to strong alkaline phosphatase activity on average in 11% of the colonies (range, 0%–20%), in IMR-90 cocultures in 35% (range, 20%–58%), and in control cultures (T84 alone) in <1% (range, 0%–2%) (Table 1). The alkaline phosphatase activity is confined to luminal formations

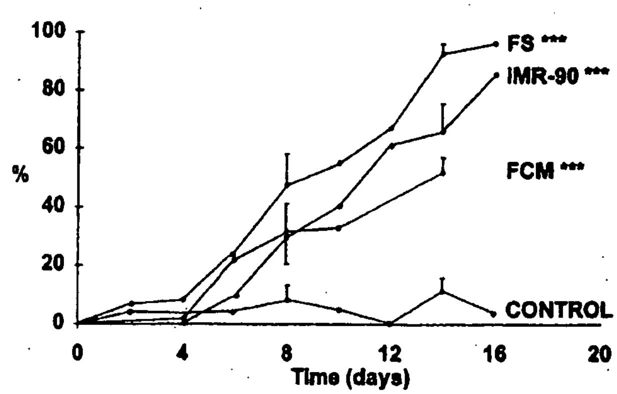


Figure 2. Mean curves for the time course of the T84 cell organization. Epithelial cells were cocultured with IMR-90 or FS fibroblasts or with FCM and without any fibroblast support (control). The amount of organized structures is presented as percentage of the total number of colonies. ***Statistical significance ($P < 0.001$) vs. control; the *bars* represent ±SEM at days 8 and 14.

with columnar but not with cuboidal T84 cells (for IMR-90, mean of 35% vs. 1%; $P < 0.001$). A typical example of moderate/strong alkaline phosphatase staining at the apical site of differentiated type of luminal formation is shown in Figure 5.

To determine whether the differentiated columnar cells in the luminal formations represent small bowel–type enterocytes or colonocytes, we studied the brush border mucin profiles of the cells. The staining showed that the differentiated columnar T84 cells in the luminal formations were negative for sulfomucin, with the mucin predominantly present in the colon. In contrast, these differentiated cells showed at apical sites sialomucin staining, as did the villus tip epithelial cells in normal human duodenal sections (Figure 6*A* and *B*). This staining was negative in luminal formations with undifferentiated cuboidal T84 cells and in the crypt cells of human duodenal mucosa.

T84 epithelial cells in organized luminal formations did not stain positively with antiserum against sucrase-isomaltase in paraffin sections, although it was detected in control stainings using normal human duodenal biopsy specimens (data not shown).

In T84 epithelial cell-fibroblast collagen gel cocultures, the basement membranes were well deposited in both the undifferentiated and differentiated types of luminal formations. This was clearly shown by the silver

Figure 3. Light microscopy of the thin sections of the fibroblast-supported T84 colonies shows two distinct types of structures: a large lumen (★) lined by a thin one-cell layer of cuboidal epithelial cells and thick-walled colonies with a small lumen and a one-cell layer of large columnar epithelial cells with basally situated nuclei (*arrows*) (hematoxylin staining; *bar* = 40 μm).

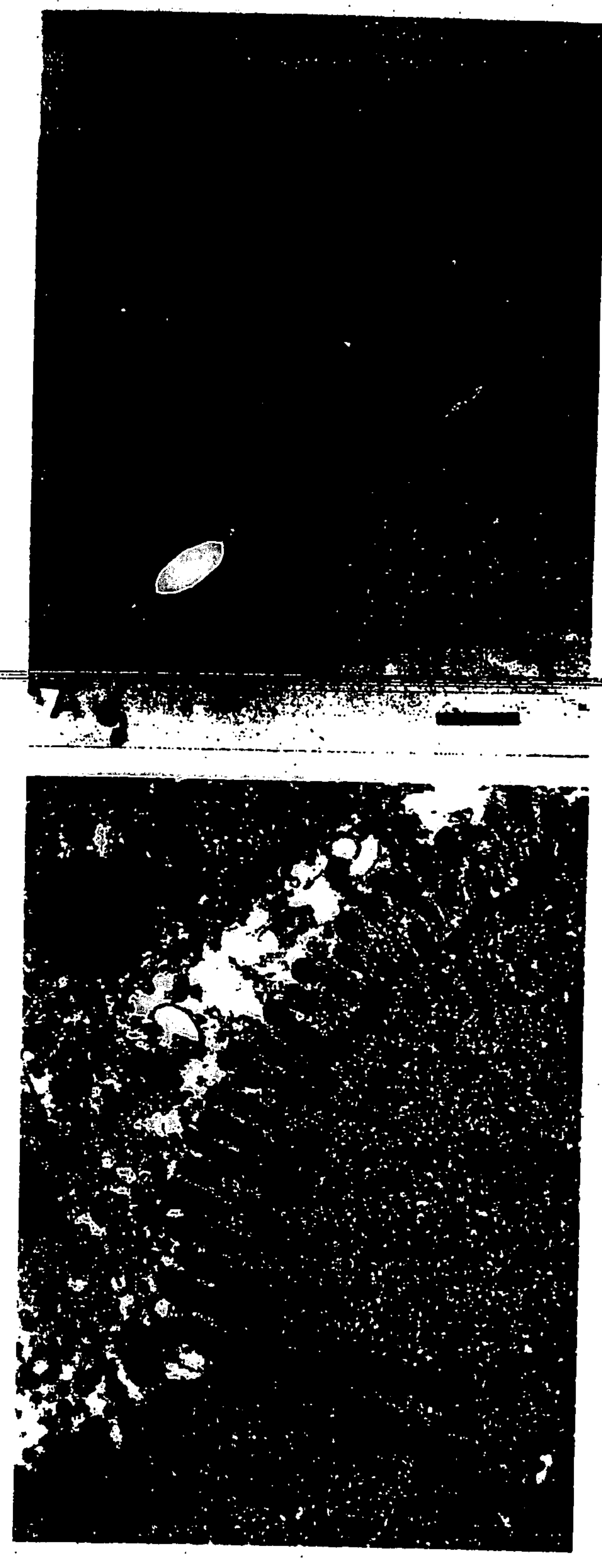
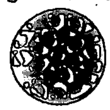
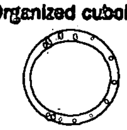


Figure 4. Electron microscopy shows that (*A*) the thin epithelial cells are cuboidal with occasional irregular microvilli (*bar* = 1 μm) and (*B*) the enterocyte-like epithelial cells are columnar and have well-developed microvilli facing the lumen (*bar* = 500 nm).

Table 1. Intensity of Alkaline Phosphatase Staining in Different Colony Types Expressed as Mean Percentages of Total Colony Counts

		Unorganized cuboidal 				Organized cuboidal 				Organized columnar 				
		−/+		++/+++		−/+		++/+++		−/+		++/+++		
Culture	No. of independent experiments	%	SD	%	SD	%	SD	%	SD	%	SD	%	SD	Total (%)
T84 (control)	15	93	6.0	0		4	4.0	0		3	2.4	0		100
T84 + FS	11	6[a]	6.3	0		48[a]	11.8	4	4.6	31[a]	5.8	11[a]	5.9	100
T84 + IMR-90	11	19[a]	10.0	0		29[a]	10.8	1	1.4	16[a]	2.3	36[a]	14.0	100

NOTE. −/+ and ++/+++ denote non/slight or moderate/strong intensity of alkaline phosphatase staining. Values represent the mean and SD percent of colony types (number of colonies examined per well was ≥50).

[a]$P < 0.001$ vs. T84 cultures with no fibroblast support.

impregnation staining (Figure 7*A*) and by immune staining for laminin (Figure 7*B*). Without fibroblastic support, only some spot-like irregularly situated basement membrane staining was seen within the T84 cell clusters.

In contrast to T84 cells, HT-29 intestinal epithelial cells failed to organize in the present three-dimensional fibroblast-epithelial cell coculture system. The unorganized round cell clusters were similar to T84 control cultures. Only T84 cells were used in additional experiments.

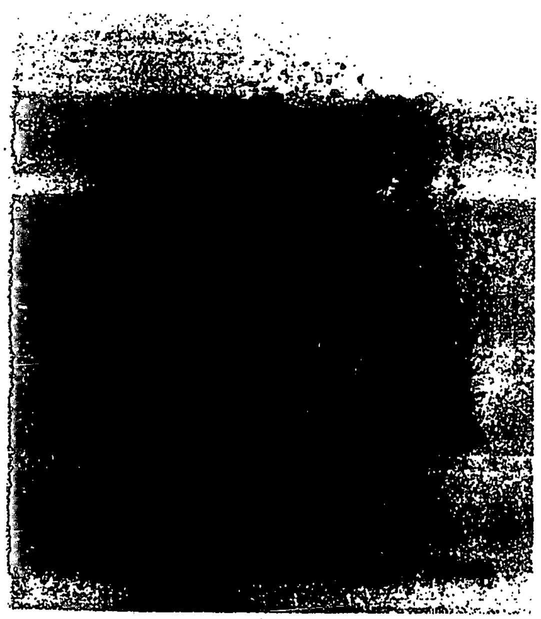


Figure 6. A typical moderate/strong alkaline phosphatase staining (*arrow*) of a differentiated T84 colony cocultured with IMR-90 fibroblasts. Apical reaction indicates that epithelial cells may have an absorptive function (*bar* = 20 μm).

Effects of HGF and TGF-β1 on the Three-Dimensional Behavior of T84 Cells

To study whether HGF was involved in the organization and/or differentiation of T84 cells, we first immunostained the cells for the HGF receptor c-met. The receptor was highly expressed in the unorganized T84 cell colonies that were not given fibroblast support (Figure 8*A*). The receptors were mainly situated on the cell membrane at the basolateral site. When T84 cells were cocultured with fibroblasts, the receptor was clearly down-regulated because the staining intensity was markedly reduced in all the luminal formations (Figure 8*B*). Next we added hHGF at 20 ng/mL to the culture medium in the absence of fibroblasts. hHGF did not induce three-dimensional T84 epithelial cell organization within the gels. In contrast, the cell colonies were clearly larger and more irregular than those seen in control cultures, indicating an increased proliferation rate. This was confirmed in the CellTiter96 AQ_{ueous} Non-Radioactive Cell Proliferation Assay because hHGF at 20 ng/mL increased T84 cell proliferation 2.4-fold from that observed in control cultures (Figure 9).

The effect of hTGF-β1 on T84 cells, grown in three-dimensional collagen gel, was tested. Different concentrations of hTGF-β1 were added to the culture medium instead of fibroblasts. With added hTGF-β1, the number of differentiated type of luminal formations increased dose-dependently within a week (Figure 10). Organization into morphologically differentiated luminal formations reached its maximum (mean, 65%; range, 63%–67%; colonies counted in two wells of three independent experiments) at an hTGF-β1 concentration of 20 ng/mL. Further doubling of the hTGF-β1 concentration to 40 ng/mL reduced the luminal structures to the same level

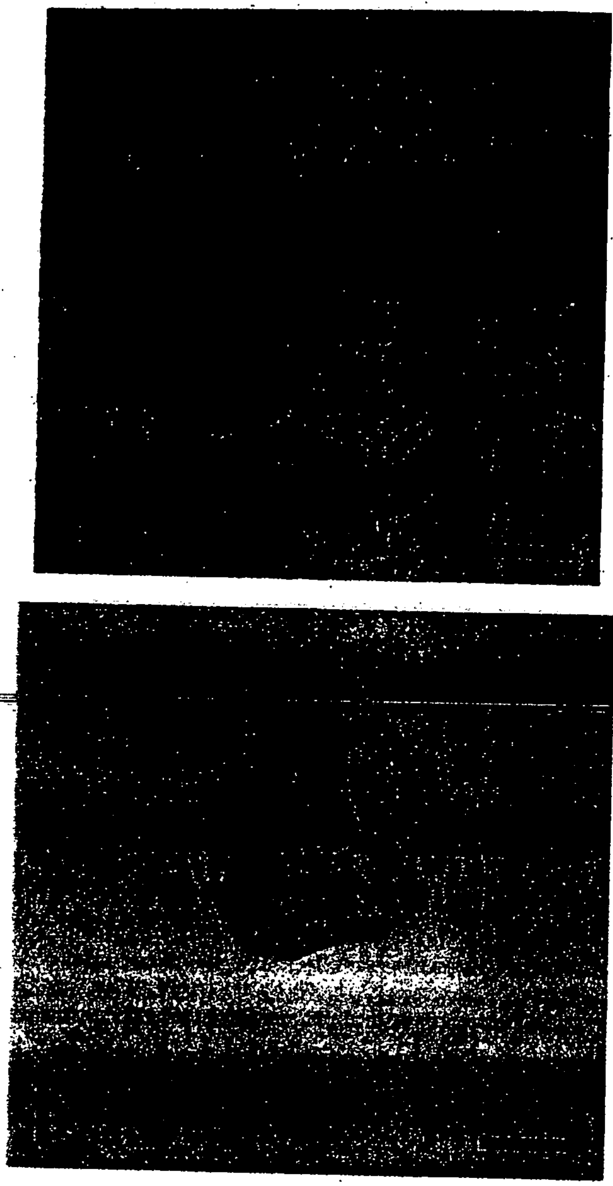


Figure 6. High-iron diamine–alcian blue staining shows sialomucins (*blue*) on (*A*) normal human duodenal villus tip brush border and (*B*) on the apical surface of enterocyte-like differentiated T84 cells (*bar* = 20 μm).

as observed at a concentration of 10 ng/mL (at 40 ng/mL; on average, 57%; range, 54%–60%; 50 colonies counted from duplicate wells in five independent experiments). These morphologically differentiated luminal formations represented 93% of organized colonies in the hTGF-β1–containing cultures. The mean count of organized colonies in the accurate controls was 6% (range, 3%–9%) of the total colony count. Alkaline phosphatase activity on the apical site of the organized and morphologically differentiated T84 cells was strong, indicating functional differentiation as well. In the proliferation assay, hTGF-β1 slightly reduced the proliferation rate of T84 cells (Figure 9).

Effects of Neutralizing Antibodies Against HGF, TGF-β, and LAP on Fibroblast-Induced Three-Dimensional Behavior of T84 Cells

To further elucidate the possible role of HGF in the fibroblast-supported three-dimensional organization of epithelial cells, the cocultures were blocked with antibodies against hHGF. The T84 cell organization was not regulated by HGF because the number of organized colonies was as in controls (on average, 59% vs. 61%). Antibodies against hHGF slightly reduced the diameter of unorganized T84 epithelial cell colonies from 72 μm (SD, 29 μm; duplicate wells in five independent experiments, with at least 50 colonies per well counted) to 61 μm (SD, 27 μm; $P < 0.05$), thus indicating inhibition of proliferation.

To determine whether the fibroblast-induced differentiation of T84 cells was caused by TGF-β, both panspecific neutralizing antibodies against TGF-β and antibodies against LAP were used to test possible suppression of the formation of the differentiated type of luminal formations. Irrelevant rabbit antibodies against human IgG served as control, with no effect on luminal formation distribution. By adding TGF-β antibodies to the cocultures, the percentage of fibroblast-induced differentiated-type colonies was reduced from the accurate control level of 42% to 15% ($P < 0.001$; Table 2). The antibodies against LAP were even more effective in blocking the formation of differentiated type of luminal structures, reducing it to 6% of all colonies. The overall colony type distribution was identical to that seen in control experiments without fibroblastic support (Table 2).

In addition, the use of antibodies against TGF-β and LAP in the cocultures seemed to increase the proliferation of T84 cells. The diameters of unorganized cell clusters increased from a mean of 90 μm (SD, 56 μm; duplicate wells in five independent experiments, with at least 50 colonies per well counted) to 111 μm (SD, 51 μm; $P < 0.05$) in TGF-β–blocked cultures and to 120 μm (SD, 50 μm; $P < 0.01$) in cultures to which antibodies against LAP were added.

Discussion

Many studies have shown that constituents of the extracellular matrix[23,24] and cell-to-cell interactions[3] make important contributions to epithelial cell differentiation, but it has also been shown that many peptide

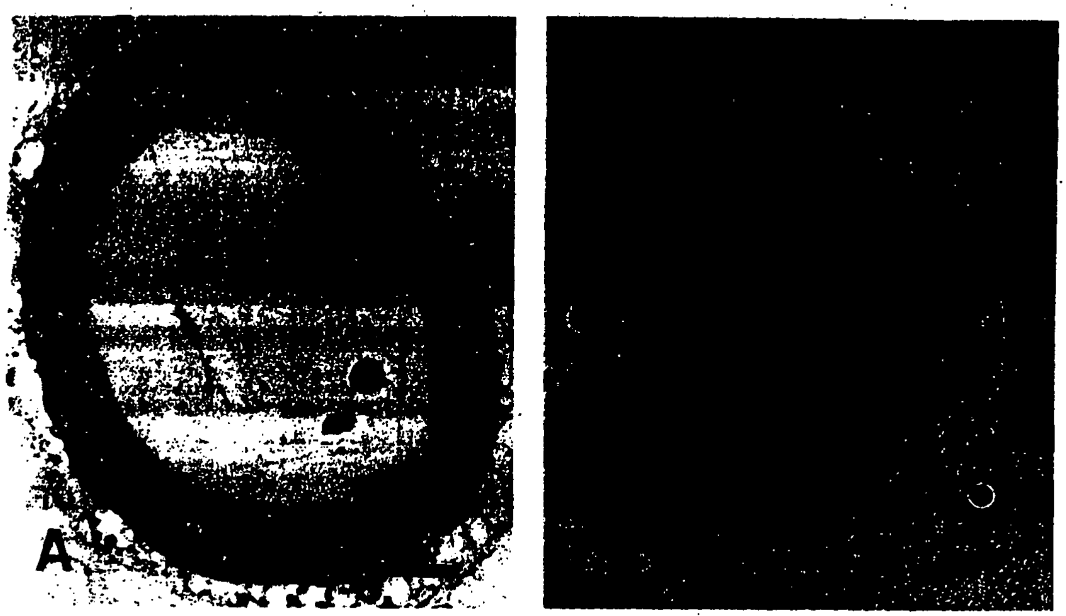


Figure 7. Organized T84 cells cocultured with fibroblasts show clear basement membrane formation (*arrows*) by (*A*) silver impregnation staining and (*B*) immunohistochemical staining of laminin (*bar* = 25 μm).

growth factors are essential.[6] Signals transmitted from mesenchyme to epithelia or vice versa in the form of growth factors constitute the basis of reciprocal mesenchymal-epithelial interactions, which are thought to play a vital role in epithelial cell growth and differentiation.[25] To study fibroblast-guided morphological changes and differentiation of intestinal epithelial cells, we used a three-dimensional coculture system with transformed intestinal cell lines, HT-29 and T84. Both of these cell lines are commonly used in studies of intestinal epithelial cell biology.

The present study, using a three-dimensional coculture system, showed for the first time that the crypt-like secretory human gut epithelial cell line T84 organizes and differentiates when given fibroblast support or TGF-β1. Furthermore, we show that fibroblast-induced T84 differentiation is mediated via TGF-β.

It has been shown that MDCK cells are able to polarize and form tubular structures when embedded in crude rat tail type I collagen gels.[10] In our model, using similar test conditions, human T84 intestinal epithelial cells were not able to form luminal structures without fibroblast support, but in fibroblast cocultures these cells responded with three types of colonies, globule clusters, and two types of three-dimensionally organized lumenal formations. The cuboidal cells in unorganized cell clusters and in organized luminal formations corresponded to the subtypes of the cell lineage that have been reported to exist in monolayer cultures.[18] We also observed a third colony type in which the T84 crypt cells turned out to be fully differentiated. The differentiation was described with the same criteria as for Caco-2[26,27] and for IEC-6[13] cells. In addition, both types of luminal formations presented occasional goblet cells (data not shown), which is in agreement with the results of McCool et al.[19] HT-29 cells, in contrast to T84 cells, failed to form luminal structures when grown three-dimensionally with IMR-90 or FS fibroblasts. In monolayer cocultures with IMR-90 fibroblasts as feeder layer, the HT-29 cells were able to grow in lumen-shaped formations (data not shown). The latter result is in agreement with observed lumen formation in monolayer cultures in the presence of HGF,[28] a factor that IMR-90 cells are known to produce.[29]

In previous studies, the cooperation of mesenchymal and epithelial cells in basement membrane formation has been clearly shown,[30,31] and it has been assumed that straight epithelial-mesechyme cell-to-cell contact is needed. However, our study shows that no cell-to-cell contact is needed for silver stain–positive basement membrane formation or laminin deposition because the present coculture method allows no straight cell-to-cell contact. The basement membrane was well deposited around both types of luminal formations, indicating the importance of basement membrane for organized epithelial cell polarization, which is most probably the first step to be taken in epithelial cell differentiation.

The forming of tubular structures by MDCK cells have been shown to be stimulated by HGF and inhibited by TGF-β.[15] Intestinal T84 cell line behaved clearly dif-

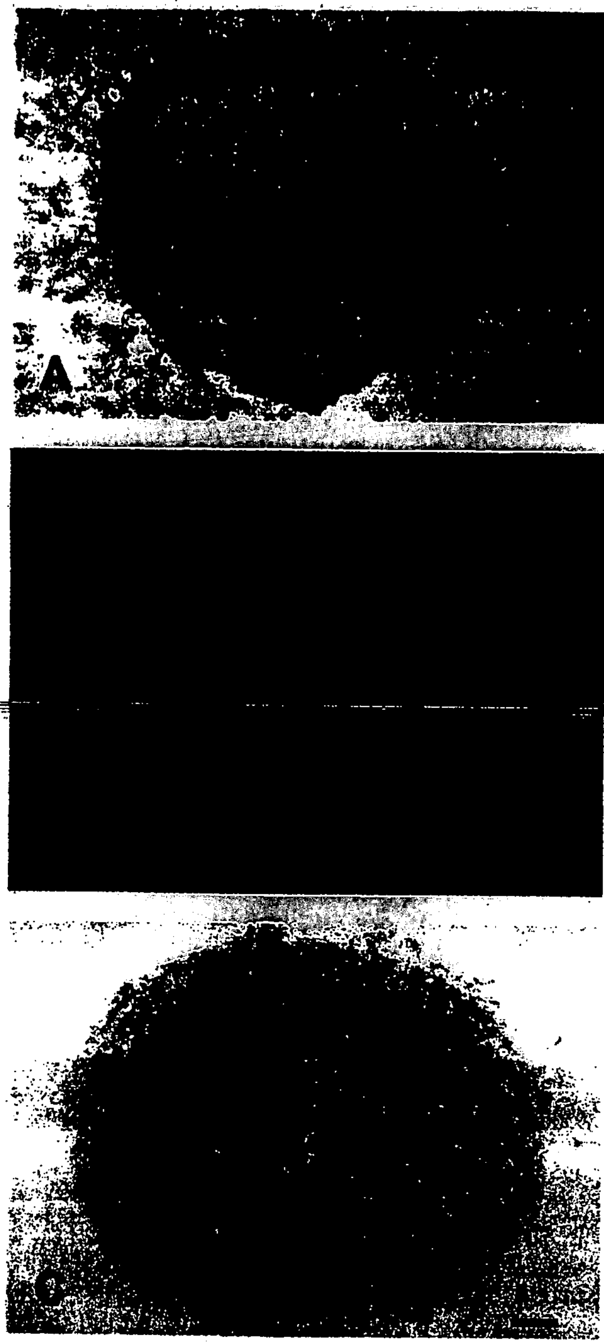


Figure 8. Immunohistochemical staining of the HGF receptor c-met. (*A*) A T84 cell colony cultured without fibroblast support shows strong red staining of c-met, (*B*) whereas in a colony cultured with IMR-90 fibroblasts, c-met is far less markedly stained. (*C*) The specificity of staining was confirmed by blocking the c-met antiserum with h-met peptide (*bar* = 20 μm).

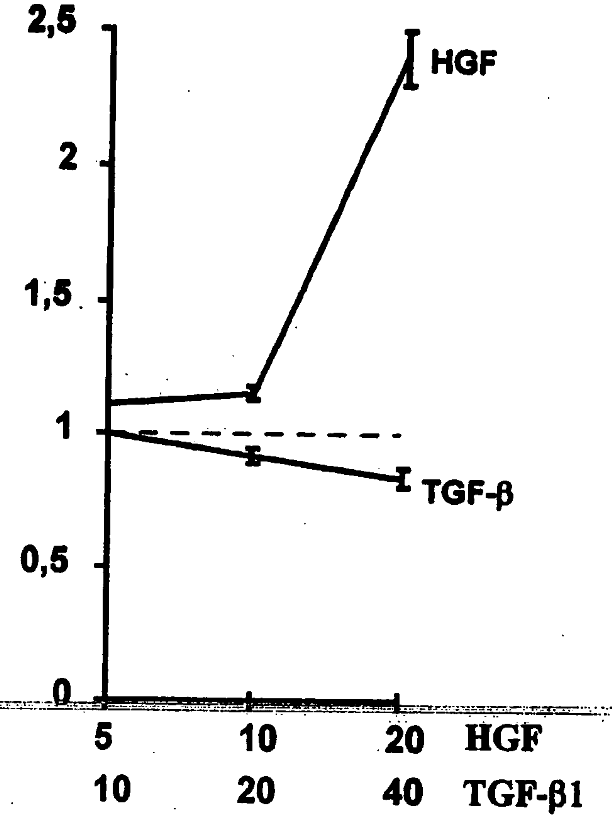


Figure 9. Effect of different concentrations of hHGF (5, 10, and 20 ng/mL of medium) and hTGF-β (10, 20, and 40 ng/mL of medium) on the proliferation of T84 epithelial cells cultured for 2 days with the growth factors. The results are arbitrary values compared with control culture without any added growth factors.

ferently from the MDCK cells, and the organization and differentiation of the cells in our three-dimensional cell culture system was shown to be caused by TGF-β. When hTGF-β1 substituted the fibroblasts in the present cocultures, the number of globular luminal formations with differentiated epithelial cells was highly elevated. Anti–TGF-β and anti-LAP antibodies blocked the fibroblast-induced differentiation as well.

TGF-β seems to be a multifunctional growth factor.[32] Our results suggest that TGF-β regulates the transition from undifferentiated mitotically active epithelial cells to nondividing enterocytes committed to terminal differentiation. The first step in this pathway might be the inhibition of proliferation by down-regulation of receptors contributing to positive proliferation signaling, as seems to be the case with c-met expression.[33] The second step is modulation of extracellular matrix composition,[34] which in turn regulates epithelial cell differentiation.[35]

HGF and its receptor c-met[36] represent a paracrine signaling system involved in mesenchymal-epithelial cell interactions during development and morphogenesis. In the present study, HGF did not induce the organization of T84 cells but increased the proliferation rate instead. This is in contrast to the finding by Nusrat et al.,[7] possibly because in our experiments HGF was served at the basolateral site, where its receptor c-met is also situated.

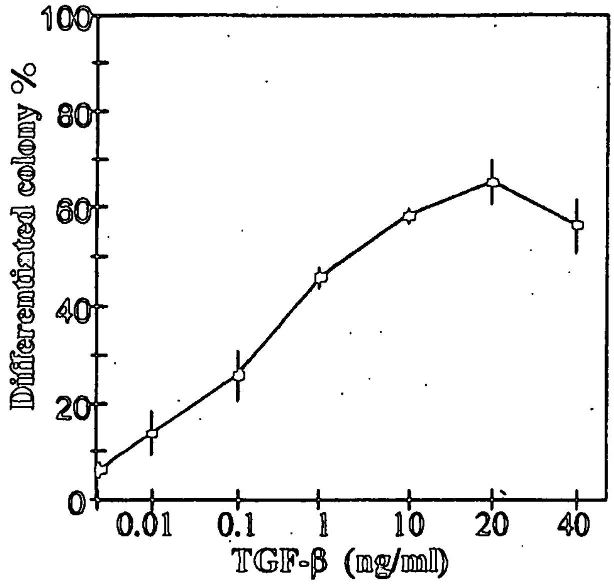


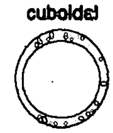
Figure 10. Dose effect of TGF-β on the three-dimensional colony differentiation of T84 cells cultured in collagen gel without fibroblasts.

C-met was highly expressed on T84 colonies grown within collagen gels without fibroblast support or added growth factors. Interestingly, when T84 cells were given fibroblast support, the receptor expression was down-regulated because there was only slight if any staining seen around the vesicles with differentiated epithelial cells. This result was unexpected because the expression of c-met on malignant cells has been stated not to change[37] and may be considered as additional evidence of T84 cell differentiation.

The present study clearly shows that the deep-crypt secretory T84 cells undergo structural differentiation and the differentiated columnar epithelial cells also up-regulate brush border alkaline phosphatase, an enzyme frequently used as a functional marker of cell differentiation in colon cancer cells.[27] Further, the mucine profiles of the differentiated T84 cells in luminal formations suggest the cells to be more like small intestinal enterocytes than colonocytes. The mucins vary in composition and relative amounts in the small intestine and colon, with more sialomucin found in the small intestine and more sulfomucin in the colon. However, we failed to show up-regulation of sucrase-isomaltase, a small bowel–specific marker, in our paraffin-embedded gel blocks. In this respect, T84 cells differ from Caco-2 cells.[26] On the other hand, T84 cells were studied after being cultured three-dimensionally for 10–14 days in gels. The sucrase-isomaltase activity increases during culture in Caco-2 cells, being still very low at day 9 when the cells are already structurally differentiated and reaching a level of 50% of that found in human small intestine only after 19 days in culture.[26]

We conclude that organization and differentiation of the secretory crypt-like T84 intestinal epithelial cells in the present three-dimensional coculture was induced by fibroblast secretion products, especially TGF-β1. The present in vitro model may prove to be the method for studying the gene products that regulate the extracellular matrix-mesenchymal cell-epithelial cell cross-talk occurring in the human jejunum on the mucosal crypt-villus axis, where the chloride-secreting crypt cells proliferate and differentiate to absorptive villus tip enterocytes.

Table 2. Distribution of Growth Factor–Induced Three-Dimensional Organization and Differentiation of T84 Cells Expressed as Mean Percentages of Total Colony Counts in T84 Cultures and in Cocultures With IMR-90 Fibroblasts

			Unorganized cuboidal 		Organized cuboidal 		Organized columnar 		
Cells in culture	Growth factor	Antibody	%	SD	%	SD	%	SD	Total (%)
T84	—	—	93	8.4	1	1.6	6	7.8	100
T84 + IMR-90	—	—	39[a]	7.6	19[a]	7.6	42[a]	5.1	100
T84	HGF	—	93	2.4	0		7	2.4	100
T84	TGF-β	—	40[a]	4.4	3	3.0	57[a]	2.5	100
T84 + IMR-90	—	anti-HGF	41	6.1	9	8.0	50	10.8	100
T84 + IMR-90	—	anti-TGF-β	79[b]	8.8	6[b]	6.8	15[b]	3.3	100
T84 + IMR-90	—	anti-LAP	85[b]	4.4	7[b]	4.1	8[b]	4.0	100

NOTE. Values represent the mean and SD percent of colony types in five independent experiments. At least 50 colonies in duplicate wells were counted.

[a] $P < 0.001$ vs. T84 cultures with no fibroblast support.

[b] $P < 0.001$ vs. untreated T84 and IMR-90 coculture.

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ABSTRACT OF PAPER

HEPATOCYTE GROWTH FACTOR ENHANCES INTESTINAL MUCOSAL CELL FUNCTION AND MASS *IN VIVO*

Yoshifumi Kato, MD, Dahong Yu, PhD, Jeff R. Lukish, MD and Marshall Z. Schwartz, MD, FAAP. Childrens Hospital, Washington DC 20010.

Hepatocyte growth factor (HGF), originally known to stimulate hepatocyte DNA synthesis, recently has been shown to stimulate growth of intestinal epithelial cells *in vitro*. However, there have been no studies on the effect of HGF on the function of intestinal epithelial cells *in vivo*. This study was designed to examine the effect of systemically administrated HGF on intestinal epithelial cell mass and function.

Methods: Twenty young adult male Sprague-Dawley rats had placement of jugular venous catheters connected to subcutaneously placed osmotic minipumps. The rats were divided into 4 groups based on the contents in the osmotic pump: Group 1 (control, n=5) normal saline; Group 2 (n=5) HGF 75 μg/kg/d; Group 3 (n=5) HGF 150 μg/kg/d; and Group 4 (n=5) HGF 300 μg/kg/d. Following a 14 day infusion, [C^{14}] galactose and [C^{14}] glycine absorption were measured in a 10 cm segment of mid small intestine using an in vivo closed-recirculation technique. Mucosal DNA content and protein content of the same small bowel segment were determined for each group.

Results: The results for mucosal substrate absorption, DNA content and protein are shown below.

	Control	Group 2	Group 3	Group 4
Galactose(μM/cm^2)	1.21	2.07(↑170)**	2.39(↑197)**	1.86(↑153)**
Glycine (μM/cm^2)	1.62	2.27(↑140)*	1.97(↑122)**	1.79(↑111)
DNA($\times 10^{-3}$ μg/mg)	103	186 (↑180)*	264 (↑255)**	288 (↑279)**
Protein(μg/mg)	42.6	56.4 (↑133)*	64.2(↑151)**	60.4(↑142)**

** $p<0.01$, * $p<0.05$, ( )=% of control

Conclusions: These data demonstrate for the first time that HGF can increase intestinal epithelial cell function and stimulate cell proliferation *in vivo*. HGF may be clinically useful in patients with Short Bowel syndrome.